Testing expectations of connectivity and breeding biology among shark species in a tropical hot spot: the Indo-Pacific.

by

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Abstract

Delineating the extent of connectivity for populations of marine megafauna and understanding the elements driving observed patterns of genetic structure is pivotal for defining the scale of management required. Many species of reef associated sharks have discontinuous distributions separated by vast expanses of unsuitable habitat. The extent of connectivity for many species of sharks throughout the Pacific and Indian Oceans is currently unknown.

The Indo-Pacific region is considered a hotspot for tropical sharks, with the majority of all reef shark species found in its waters. Many nations including Indonesia, Australia and Papua New Guinea exploit shark and ray populations as either target or bycatch at varying catch levels. Information on fisheries is better known for Indo-Pacific locations Indonesia and Australia while little is known of their neighbouring nation Papua New Guinea.

Genetic markers in the form of mitochondrial DNA (mtDNA), microsatellites (Msats) and Single Nucleotide Polymorphisms (SNPs) can be used to infer a species' biology and ecology. These genetic markers vary in inheritance mode, location within the genome and likelihood of being affected by cellular mechanisms such as recombination. Theoretically, genetic markers have varying capacity to reveal population-level differences and therefore delineate stock structure and connectivity. Such differences mean markers can illuminate processes at different points along the evolutionary trajectory of populations. Often genetic markers are used interchangeably and no formal testing of stock assignment using all three marker types has occurred for any shark species.

This thesis draws together diverse genetic approaches to generate novel insights into (i) shark ecology across the Pacific and Indian Oceans, (ii) breeding behaviour of two shark species and (iii) empirical comparisons of genetic diversity and population connectivity using multiple genetic markers.

The thesis is composed of a general introduction (Chapter 1), two population genetic studies, each on a commercially important species of shark (Chapter 2-3), a multiple paternity assessment (Chapter 4) and a final discussion and conclusion (Chapter 5). Population genetic studies are reported here for the silvertip shark (*Carcharhinus albimarginatus*) (Chapter 2) and the scalloped hammerhead shark (*Sphyrna lewini*) (Chapter 3) to better understand their connectivity throughout the Pacific and Indian Oceans. The genetic differences measured between populations sampled in this study revealed that large body size and ability to cross vast expanses of open ocean was not a consistent predictor of the genetic cohesiveness of the species. The fourth chapter assesses the presence and prevalence of multiple paternity in litters of female grey reef shark (*C. amblyrhynchos*) and *S. lewini* individuals captured in Papua New Guinea. Finally, Chapter 5 synthesises these new

insights for management purposes and critically compares the variety of genetic markers deployed and identified key considerations for elasmobranch researchers before designing future population genetic studies.

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"Words are, in my not-so-humble opinion, our most inexhaustible source of magic."

-Albus Dumbledore

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References:

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Statement of co-author contributions

The following people contributed to the publication of the work undertaken as part of this thesis:

Paper 1/ Chapter 2- *Novel multi-marker comparisons address the genetic population structure of silvertip sharks (Carcharhinus albimarginatus)*

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- Sharon Appleyard (CSIRO) (10%)
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Paper 2/ Chapter 4- *Variability in multiple paternity rates for grey reef sharks (Carcharhinus amblyrhynchos) and scalloped hammerheads (Sphyrna lewini).*

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Madeline Green contributed with developing thesis ideas, data analysis and completing manuscript writing.
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Sean Tracey contributed with project development and development and refinement of manuscripts.
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Contents

List of Figures

List of Tables

Chapter 1 General Introduction

1.1 Measuring connectivity in marine ecosystems

The movement of organisms between regions is critical for population persistence and is key for sustaining the ecological integrity of ecosystems (Cowen et al., 2006; Beger et al., 2010). The understanding of movement is directly used in conservation and management planning to ensure long-term survival of a species throughout its range (Mills et al., 1996). Estimating patterns of connectivity for marine species is challenging due to the general lack of physical barriers present within species distributions. Despite these complexities, movement patterns of marine species have been well documented across a number of taxa including molluscs, crustaceans, fishes and marine mammals (Gillanders et al., 2003; Palumbi, 2003; Cowen et al., 2006; Toonen et al., 2011). Often alternative structural and bio-physical drivers are shown to significantly affect connectivity in marine ecosystems. Depth, salinity, precipitation, oxygen and turbidity are often found to drive marine species movements (Selkoe et al., 2016).

Connectivity can be defined a number of ways across spatial and temporal scales, i.e. structural, functional, demographic and genetic connectivity (see Selkoe et al., 2016 for review). Demographic and genetic connectivity directly relate to the dispersal of individuals and genes respectively, and the effects on population-level processes (e.g. growth, mortality) (Lowe et al., 2010; Selkoe et al., 2016). These measures of biological connectivity are mainly concerned with movements made by individuals between habitats diurnally, seasonally or during their life cycle for reproduction or feeding (Beger et al., 2010). Since genetic connectivity describes the movement of genes and accounts only for individuals that have successfully reproduced after dispersing, often estimates span more than one generation (Mills et al., 1996; Lowe et al., 2010; Selkoe et al., 2016). Therefore, genetic connectivity regularly reflects dispersal over long time spans and can represent historical connectivity (Wright, 1951; Rieseberg et al., 2008; Epps et al., 2015). Understanding and maintaining genetic connectivity for a species between regions is important in long-term spatial management planning and stock assessment for fisheries and conservation management.

1.2 The role of genetic and genomic techniques for fisheries management

The use of genetic tools to assist and inform fisheries management has been occurring for over 50 years (Ryman et al., 1987). Genetics enables researchers to identify a variety of biological information important for fisheries assessments (Ovenden et al., 2015). Such information includes species identifications, estimates of stock structure and genetic health, calculation of effective population sizes and sex-biased behaviours (see Ovenden et al., 2015 for full review). Genetics can also study the parentage in natural populations using DNA profiling methodologies (Birkhead et al., 1998). Arguably the most important genetic measurements used in fisheries management are estimates of the spatial extent of a biological stock (also referred to here as a genetic stock or population). The level of connectivity identified within and between populations can define the geographic boundary of a biological stock (Ovenden et al., 2009). In general, a biological stock refers to a species in a given geographic area with limited interbreeding with other biological stocks of the same species (FRDC, 2018). A challenge for fisheries managers often appears when a single biological stock occurs over a number of jurisdictions and is therefore subject to several fisheries. Where possible it is important to assess each biological stock separately in order to understand the combined exploitation or pressure occurring across jurisdictional boarders.

Genetics provides a number of markers (target sections of DNA, also called loci- plural or locus - singular) and technologies to answer questions specific for fisheries management. These markers locate portions of DNA from the mitochondrial (mtDNA) and nuclear genomes (nDNA). MtDNA genes are maternally inherited while nDNA is bi-parentally inherited producing information from both mother and father (Nei, 1975). Because of its characteristics (no recombination, maternally inherited) mtDNA represents an evolutionary population signature ideal for identifying phylogenies and historic population expansion and bottleneck events (Avise et al., 1998), but is useful for population structure analyses also. Alternatively, nDNA often in the form of microsatellite markers (Msats) or Single Nucleotide Polymorphisms (SNPs) can answer more contemporary questions of population structure, making these highly suitable for biological stock structure analysis (Ovenden et al., 2015). Additionally, when comparing mtDNA and nDNA, differences in male and female gene flow can be investigated (Prugnolle et al., 2002). Other, more novel nDNA approaches include genome-wide analyses identifying Single Nucleotide Polymorphisms (SNPs) located in coding and non-coding regions of the genome (Morin et al., 2004).

The choice of genetic marker requires critical consideration when designing population genetic studies as compromise must be made between the amounts of genetic data that can be obtained, the amount of genetic data required and project costs (Hodel et al., 2017). There are both

advantages and disadvantages for using Msat or SNP markers for biological stock structure and connectivity estimates. Until recently Msats have been the marker of choice, meaning often primers, genotyping analyses and software packages are optimised and available for use (Seeb et al., 2011; Hodel et al., 2016). The addition of individual samples and/or loci is relatively simple and cost effective with Msats. However, if Msat primers are not yet developed for the species of interest, the project costs and time spent optimising can be significant and as expensive as current high-throughput sequencing for SNP discovery (Hodel et al., 2016). However, Msats are usually limited in the number of loci (< 30) and are prone to bias such as homoplasy (parallel evolution of identical loci) (Morin et al., 2004). SNPs are increasingly being used for population genetic studies as thousands of loci can be generated from hundreds of individuals in a matter of weeks. SNPs are considered a reduced representation of a whole genome providing a significant number of loci at a fixed cost and timeframe. SNPs also have a number of biases due to issues encountered in the wetlab and during bioinformatics processing including sequencing errors, incorrect genotype calling and misassembly of paralogous reads (Etter et al., 2011). Additionally, if no reference genome (annotated map of protein coding genes) is available, loci obtained are essentially anonymous and unable to be mapped to coding or non-coding regions of the genome (i.e. linked to adaptive/neutral genes) (Andrews et al., 2014, 2016). Often reference genomes aren't available (currently only 0.1% of all vertebrate genomes are sequenced) as they require extensive sequencing across the whole genome, complex assembly and then annotation to previously characterised genes (Ellegren, 2014).

Biological stock assessments for fisheries management purposes using either Msats or SNPs are often being undertaken interchangeably with little understanding of the possible differences in interpretation between markers. The irregular use of genetic markers for biological stock assessments is a growing concern considering new genomic markers (such as SNPs) are now common for connectivity studies. It is important to identify which genetic markers should be used when answering biological stock assessment questions (see review- Ovenden et al., 2015). Ovenden et al., (2015), however, do not cover how new genome-wide approaches will be best used in fisheries management. Employing multiple genetic approaches (e.g. mtDNA, Msats and SNPs) would allow varying resolutions of biological stock structure to be measured, providing evidence of the benefits and/or shortcomings of each marker type. Furthermore, having a greater number of individual genetic markers reduces bias and sampling error in statistical models commonly used for analysis (Waples, 1998).

1.3 Connectivity and ecology of sharks

Molecular genetic approaches provide a unique tool for the study of elasmobranchs (sharks and rays) (Dudgeon et al., 2012). Sharks do not have pelagic eggs and larvae, therefore connectivity and the boundaries of their biological stock structure is largely dependent on adult movement (Carrier et al., 2004). Low levels of genetic differentiation is sometimes found among populations due to high gene flow between distinct locations for shark species (Hoelzel et al., 2006; Veríssimo et al., 2010; Daly-Engel et al., 2012; Momigliano et al., 2015; Bailleul et al., 2018; Corrigan et al., 2018). Whether genetic similarity between populations is an artefact of historic connectivity or indicative of contemporary movements remains unknown for the vast majority species (Bailleul et al., 2018). Novel genome-wide molecular methods (i.e. SNPs) produce large suites of loci suitable for accurately measuring low gene flow between populations (Kohn et al., 2006). Thus, increasingly, SNP markers are being employed to explore the shark genome more deeply and resolve biological stock structures for conservation and fisheries management purposes (Portnoy et al., 2015; Momigliano et al., 2017; Pazmiño et al., 2017, 2018; Junge et al., 2019).

Increases in fishing pressure (commercial and artisanal) raise concerns given the life-history characteristics of sharks (low fecundity, late age at maturity and long life-spans) make them vulnerable to overexploitation when fishing mortality is higher than natural mortality (Stevens, 2000; Dulvy et al., 2014). Current estimates calculate a quarter of all shark and ray species are threatened under the criteria of the IUCN Red List for threatened species with over-fishing and habitat degradation identified as the leading causes (Dulvy et al., 2014). The loss of sharks and rays in a local system can have detrimental implications. Many elasmobranchs are apex and meso-predators essential to the maintenance and stability of food webs (Stevens, 2000; Kitchell et al., 2002). While shark fishing is considered to be sustainable in some cases (Holden, 1973; Walker, 1998; Prince, 2005), many fisheries have left populations heavily depleted or in some cases locally extinct (Olsen, 1959; Rago et al., 1998; Dulvy et al., 2000; Davidson et al., 2015). Similar to teleosts, fisheries management of elasmobranchs is primarily dependant on setting restrictions based on biological estimates such as recruitment, growth and mortality rates (Stevens, Walker, & Simpfendorfer, 1997; Walker, 1998). Alternative strategies, however, include targeting younger, non-mature cohorts using size selective gear such as gillnets (Prince, 2005). Such biological and demographic estimates are generally calculated per biological stock; therefore understanding the biological stock boundaries of a population is crucial to ensure harvest rates are sustainable.

The specific mating systems of sharks are often complex involving diverse breeding strategies including monogamy, polyandry, pathogenesis and hermaphroditism (Iglésias et al 2005;

Dudgeon et al 2017; Feldheim et al., 2001; Chapman et al., 2004; Daly-Engel et al., 2006). The most well recorded of these are polyandrous behaviours whereby female sharks mate with many males during a single breeding season (Byrne et al., 2012). Polyandrous broods can contain individuals which are either full and half siblings- referred to as 'multiple paternity' (Birkhead et al., 1998). Having a single brood with multiple fathers is proposed to influence the genetic diversity of a biological stock and alters our understanding of the relative reproductive success of individuals, the maintenance of population genetic diversity and possibly the future of evolutionary potential for the entire species (Chapman et al., 2004). Studying the presence of multiple paternity is possible due to the internal fertilisation and gestation in utero of litters of offspring. Given the importance of multiple paternity (especially to a group who have no larval dispersal stage) where possible studies should be undertaken to identify the mating system.

1.4 A summary of shark fishing in Papua New Guinea

The coral triangle hosts some of the most diverse assemblages of coral reef fishes (including sharks) in the world (Veron et al., 2009). While a significant amount of work has gone into understanding catch rates of sharks and rays in Indonesia (White et al., 2006) very little is known of sharks and rays in the Papua New Guinea (PNG) region. Like many of its neighbouring nations, PNG's economy and local communities rely heavily on wild marine life as a source of income and food (Vieira et al., 2017). Commonly targeted animals in the PNG region include reef fish (Cinner et al., 2006; McClanahan et al., 2008), sea cucumbers (marketed as 'Beche-de-mer') (Kinch, 2002) and sharks/rays (Kumoru, 2003; Sabetian et al., 2006). Shark and ray products are the country's 5th most important export, contributing over 2000 metric tonnes (mt) (processed weight) of shark and ray fins and flesh to market, worth a total of ~2 million USD a year (Kumoru, 2002, 2003).

In PNG, sharks are impacted through a combination of commercial and artisanal fisheries targeting a number of species using longline, purse seine, trawl and traditional fishing gear (Cinner et al., 2006; Vieira et al., 2017). Originally, commercial fishing for sharks in PNG began in the early 1980s and within 10 years the fishery was closed due to decreasing catch rates and international sanctions on netting practices (Kumoru 2002). Despite bans on targeted shark and ray harvests, the high prices offered for their products led to unregulated fishing by licenced tuna longline vessels who collected sharks as bycatch and stored their product in on-board freezers (Kumoru 2002). Exported product for sharks and rays increased dramatically from 20 mt exported in 1990 to 2000 mt exported only ten years later in 2000. In 2002, the National Fishing Authority (NFA) in PNG

recognised shark fishing as a 'legitimate fishery that required management' and established the National Shark Longline management plan (Kumoru, 2002) for commercial fishing practices. The plan was governed under the PNG Fisheries Management Act 1998 and allowed only nine vessels with crew of PNG origin to fish commercially for sharks within the countries Exclusive Economic Zone (EEZ). Additionally, the plan ruled that fishing may occur within the EEZ of PNG however is prohibited inside six nautical miles from land, island and emergent reef (Kumoru, 2002). Despite the introduction of fishing restrictions and improved management efforts, in 2014 the shark and ray fishery was closed. The shutdown was attributed to the majority of their catch comprising of silky sharks (*Carcharhinus falciformis*), a species declared as no-take effective from the first of July 2014 (WCPFC CMM 2013-08). Currently, no target shark and ray fishery is operating within PNG, however commercial vessels (mostly tuna long-liners) remain harvesting sharks as bycatch, retaining fins and flesh.

The majority of PNG people undertake subsistence fishing activities including harvesting marine resources for food or to sell them for basic living necessities (Branch et al., 2002; Cinner et al., 2006), these fishing efforts are hereafter referred to as the artisanal fishery. For the local artisanal fishery, there is almost no information available on the landings and species composition of shark and rays. A single study completed for the Milne Bay province identified 24 different species of reef associated sharks and rays that were captured through artisanal fishing, 20% of which were considered Vulnerable or Endangered under the IUCN Red List (Appleyard et al., 2018). Recent socioeconomic analyses has indicated that the artisanal shark and ray fishery is one of the most important resources for PNG people, following beche-de-mer (BDM), which has since been closed by the NFA due to overexploitation (Butler et al., 2014; Vieira et al., 2017). The loss of the BDM fishery means local communities are depending more acutely on the shark and ray fishery as a source of income and food (Vieira et al., 2017).

The shark and ray fauna of PNG are not well understood (Last & White, 2011) and catch rates are thought to be underestimated for the region (Vieira et al., 2015). A lack of available biological and fisheries dependent knowledge reduces the ability of the NFA to identify if stocks are at risk of being overfished. Effectively managing PNG's shark and ray resource is also of interest to neighbouring countries including Australia and Indonesia. Since many sharks and rays are highly migratory in nature, connectivity between these closely located regions may be possible. However, managing a single biological stock represented within a number of national EEZ's can be challenging as the biological stock is no longer the sole responsibility of an individual country (Stevens, 2000). Instead co-management between international authorities must occur to ensure fishing effort is regulated across the entire biological stock (Ovenden et al., 2009). Given the vast differences in

fishing pressure, management and conservation efforts between PNG, Australia and Indonesia, identifying existing biological stock structure is crucial for planning long term sustainability measures for shark populations across the Indo-Pacific.

1.5 Objectives to understand shark fisheries in Papua New Guinea and greater Indo-Pacific region

In early 2014, the Australian Centre for International Agricultural Research (ACIAR) commissioned CSIRO to work alongside the PNG-NFA to assess the sustainable management of the shark resources of PNG (Project: FIS/2012/102). The large-scale project assessed the fishery by focusing on biological, economic and social factors. Through an intensive observer sampling program, the project produced a number of stock assessments for commonly caught species. Observers working on the vessels collected an array of information on both the fishery (e.g. gear deployed, catch rates, condition and fate of catch, fishing locations) and target/by-catch species (length, sex, maturity). Observers collected biological samples from many of the landed sharks and rays to be used for population genetic analyses.

This PhD project uses the genetic material sourced from the ACIAR/CSIRO/NFA project to identify the connectivity and biological stock structure of two of the commonly caught shark species in the PNG target longline fishery which are also caught in other fisheries in PNG. The two study species were harvested in large numbers and subsequently many samples were available for analyses. These species are the silvertip shark (*Carcharhinus albimarginatus*) and scalloped hammerhead (*Sphyrna lewini*). Additionally, samples from pregnant female *S. lewini* and grey reef shark *Carcharhinus amblyrhnchos* were sourced from PNG and litters were tested for multiple paternity. Throughout the PhD, a combination of molecular genetics tools have been used including mitochondrial DNA sequencing, microsatellite genotyping and nuclear genotype-by-sequencing of SNPs to assess the level and pattern of genetic structure (and diversity within PNG) in these two shark species by comparing with samples collected from location across the Indo-Pacific region. Combining multiple genetic/genomic methods provides a more robust platform for testing connectivity of sharks among regions and ocean basins.

The main objectives of the PhD are as follows:

- Develop and deploy molecular population markers in two key exploited shark species in PNG and the greater Indo-Pacific region (chapter 2 & 3)

- Develop next generation sequencing capacity for the characterisation and genotyping of commonly exploited shark species (chapters 2 & 3)
- Assess the intra-specific genetic connectivity of two exploited shark species within PNG (chapters 2 & 3)
- Determine if biological stocks of commonly captured shark species in PNG are shared with neighbouring countries such as Australia based on genetic and demographic/movement information (chapters 2 & 3)
- Utilise genetic information to address knowledge gaps in population, reproductive and connectivity status for continued effective management of common shark species within and outside the PNG region (chapters 2, 3 & 4)
- Compare and contrast the application of multiple genetic approaches to explore limitations and bias in sampling design, analyses and results pertinent for fisheries management (chapters 2, 3 & 5).

The work produced throughout this thesis has provided a number of novel findings addressing current knowledge gaps for sharks and the study of population genetics including; the first assessment of the genetic population structure of silvertip sharks and two case studies empirically comparing microsatellite and SNP markers for population assignment of sharks. These findings can be used directly to understand the genetic stock structure of studied shark species and used as a road map for marker choice for future population genetic studies.

Chapter 2 Novel multi-marker comparisons address the genetic population structure of silvertip sharks (*Carcharhinus albimarginatus*)

Accepted for publication- Marine and Freshwater Research 2019

2.1 INTRODUCTION

Defining the scale of connectivity among marine populations and identifying the factors driving the exchange of individuals is pivotal to our understanding of population dynamics (Cowen et al., 2006). Understanding how and why animals move (or remain resident) is essential for conservation ecology with such knowledge directly applied to spatial management planning (Palumbi, 2003; Espinoza et al., 2014 a). Species that display migrations across jurisdictional boundaries or beyond national jurisdictions altogether can complicate management efforts as international cooperation is required (Ovenden et al., 2015; Hays et al., 2016; Chin et al., 2017).

Genetic methods are commonly used to examine the biological stock structure and connectivity of wild species to assist management and conservation planning (Ryman et al., 1987; Knutsen et al., 2003). Genetic tools (in the form of 'markers') can uncover a variety of biological information important for connectivity and population structure estimates (Ovenden et al., 2015). Markers widely used for population genetic studies include short regions of mitochondrial DNA (Grahame et al., 1995) and nuclear microsatellite loci (Selkoe et al., 2006). Advances in next-generation sequencing technology enables screening of loci across whole genomes and multiple individuals thereby, providing geneticists access to thousands of loci commonly in the form of single point mutations referred to as Single Nucleotide Polymorphisms (SNPs) (Morin et al., 2004). SNPs are increasingly being used for population structure studies (Hess et al., 2011; Jeffries et al., 2016; Momigliano et al., 2017; Pazmiño et al., 2018; Junge et al., 2019), however their bi-allelic nature means SNPs contain less information per locus then multi-allelic microsatellites (Coates et al., 2009). Nevertheless, in studies where thousands or more SNP loci are used, they are proving powerful enough to resolve fine-scale population structure (Rosenberg et al., 2003; Morin et al., 2004; Liu et al., 2005; Rasic et al., 2014; Vendrami et al., 2017).

Depending on the marker selected, genetics can explore historic and contemporary population patterns as well as compare differences in male and female connectivity among populations (Feutry et al., 2017). One of the most important genetic measurements for marine spatial management is estimating the level of connectivity among populations. The extent of genetic subdivision identified in a population can help define the geographic boundary of a biological stock

(Nielsen et al., 2009; Lowe et al., 2010; Ovenden et al., 2015). The application of population genetics has been successful in uncovering genetic stock structures and providing robust estimates for spatial management in many marine species (Appleyard et al., 2002; Blaber et al., 2005; Salini et al., 2006; Ovenden et al., 2009; Horne et al., 2011; Pazmiño et al., 2018). The silvertip shark, *Carcharhinus albimarginatus* is one species that will benefit from connectivity assessments, due to its discontinuous distribution in the Indo-Pacific and recent global population declines (Espinoza et al., 2016). Occurring on continental shelves, offshore islands and coral reefs, *C. albimarginatus* inhabits tropical waters to depths of 800m (Bond et al., 2015). Listed as Vulnerable under the International Union for Conservation of Nature (IUCN) Red List, globally *C. albimarginatus* has undergone rapid decline in biomass of a predicted 30% over 54 years, as estimated from survey data (Espinoza et al., 2016). Declines are attributed to heavy fishing pressure from longline, gillnet and purse seine fisheries throughout its range (Bond et al., 2015).

In Australia, *C. albimarginatus* are the second most commonly sighted shark species within the Great Barrier Reef (GBR) (Heupel et al., 2009). While not targeted, it is predicted *C. albimarginatus* make up bycatch in commercial and recreational Coral Trout line fisheries along the east coast of Australia (Heupel et al., 2009) and have also been identified during examination of Illegal, Unreported, Unregulated (IUU) fishing practises in northern Australia (Marshall, 2011). In locations such as Papua New Guinea (PNG), *C. albimarginatus* along with many other species of sharks, are caught in greater numbers than in Australia (Kumoru, 2003; White, 2007). Connectivity of *C. albimarginatus* in the region is not well understood making management challenging given the differences in catch rates between Australia and PNG.

To test the extent of connectivity among regional locations and improve our understanding of genetic structure of *C. albimarginatus*, we analysed genetic variation in mitochondrial DNA (mtDNA), microsatellites (Msats) and Single Nucleotide Polymorphisms (SNPs). By using combinations of mitochondrial and nuclear DNA markers, our understanding of genetic subdivision in broadly distributed marine species has advanced rapidly (Waples, 1998; Hellberg et al., 2002). For sharks in particular, microsatellites have been a popular marker for delineating contemporary genetic structure (Keeney et al., 2003; Feldheim et al., 2007; Karl et al., 2011; Daly-Engel et al., 2012; Bernard et al., 2016). Recently, however there has been a rise in the number of studies using suites of SNPs to measure genetic variation in the nuclear genome (Momigliano et al., 2017; Pazmiño et al., 2018; Junge et al., 2019). Ongoing improvements in sequencing technology enables thousands of genome-wide SNPs to be easily screened (Baird et al., 2008; Sansaloni et al., 2011; Peterson et al., 2012) with many studies finding the informativeness and power of SNPs to be high (Rosenberg et al., 2003; Morin et al., 2004; Liu et al., 2005; Rasic et al., 2014; Vendrami et al., 2017).

Previous genetic studies of *C. albimarginatus* have primarily focused on identifying the species within fish markets (Liu et al., 2013); as such, no population genetic assessment has been undertaken for the species. In this study we collected samples from three Indo-Pacific Ocean countries; Seychelles, PNG and Australia to identify what level of connectivity or genetic stock structure was occurring between these nations; each with varied capacities for fisheries exploitation and management. The patchy and isolated distribution of *C. albimarginatus* throughout their range suggests each location could be a distinct population, in which case connectivity would be low. Based on findings from telemetry studies (Espinoza et al., 2015 a) and similar population genetic assessments of reef shark species (Vignaud et al., 2014; Pazmiño et al., 2017) we expected gene flow to be restricted between our three sampled locations.

2.2 METHODS

Sample Collection and DNA extraction

A total of 152 *C. albimarginatus* DNA samples were obtained from three locations across the Indo–Pacific (Figure 2.1). These locations were chosen to focus on the cross-jurisdictional management of *C. albimarginatus* between PNG and Australia. One distant location (Seychelles) was selected to provide contrast to the central Indo-Pacific locations. Collection from Seychelles and east Australia occurred at one and two sites respectively, while PNG samples were obtained from a number of sites throughout the Bismarck and Solomon seas (Figure 2.1). Throughout 2015–16 samples from PNG were collected on board fishing vessels, from fish markets and local villages by observers. Fisheries independent samples from Seychelles and east Australia were collected between 2013–2017 by researchers from Environment Seychelles and James Cook University, respectively. A fin clip was taken, with individuals subsequently released. For sharks landed by commercial and artisanal fishers, a piece of vertebrae chord or muscle was collected. Associated biological data were also collected for each individual including sex, total length (TL) and maturity stage.

DNA was extracted using the Wizard© SV Genomic DNA Purification system (Promega, Australia); tissue extractions were undertaken using SV minicolumns following modifications to the manufacturer's instructions (i.e. overnight tissue digestion; amount of supernatant used to elute DNA was reduced; DNA elution times increased). Total genomic DNA was eluted in DNAse free water and quantified (ng/ul) on a Nanodrop 8000 (Thermo Fisher Scientific, Australia), after which DNA concentration was standardised to 15-25ng of gDNA.

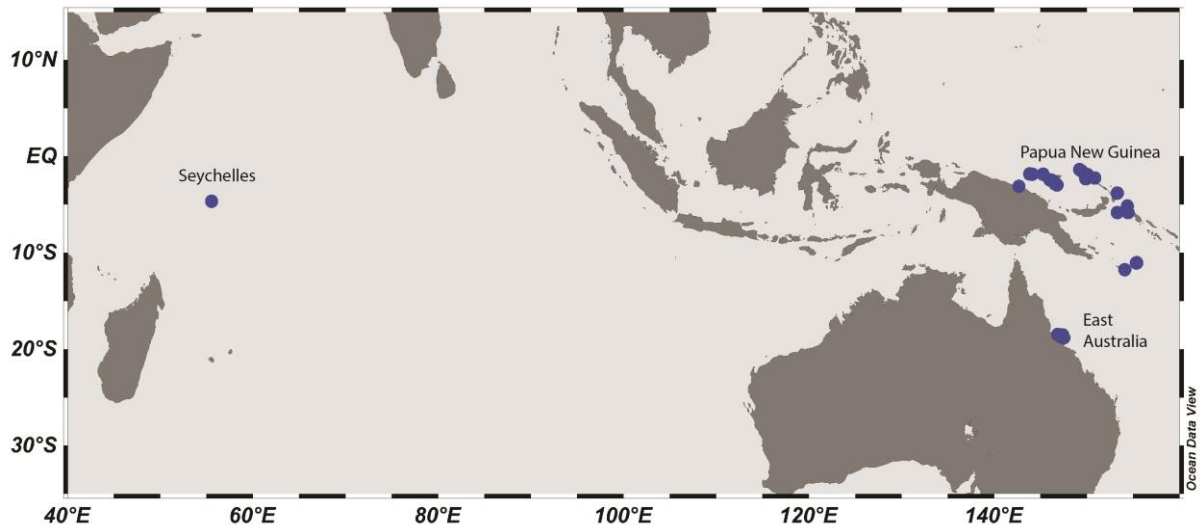

Figure 2.1. Sample collection for *C. albimarginatus* within the Indo-Pacific Ocean. West Indo-Pacific locations- Seychelles, central Indo-Pacific locations- Papua New Guinea and east Australia. Circles represent sample collection sites.

Mitochondrial DNA

To characterise similarity among and between samples from various locations we amplified 994bp of the mtDNA Control Region (CR) using the forward primer PRoL2 and reverse primer PheCacaH2 (Pardini et al., 2001). Polymerase Chain Reactions (PCR) were conducted in 25 μL reactions with 15-25 ng of gDNA, GoTaq® Green Master Mix (Promega, USA), 1 μL Bovine Serum Albumin (Promega) and 10 μM primers. PCR used the following thermocycler parameters: initial hold at 94°C/ 5 min, 35 cycles of 94°C/ 30 sec, 58°C/ 30 sec, 72°C/ 1 min, followed by final extension of 72°C/ 10 min. Successfully amplified PCR products were sequenced bi-directionally using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Invitrogen Life Technologies, USA) and an annealing stage of 58°C/5 sec for 25 cycles. Cycled sequence products were cleaned using CleanSEQ kit (Beckman Coulter, Australia) and ran on an ABI 3130XL AutoDNA sequencer (Applied Biosystems, USA) at the CSIRO marine laboratories, Hobart, Australia. Sequences were screened and aligned using Geneious v10.2.3 (Biomatters Ltd, New Zealand). We calculated molecular diversity indices including haplotype and nucleotide diversities using Arlequin v3.5 (Excoffier et al., 2010). To visualize haplotype structure between locations, Median-Joining network analysis was constructed using POPart v1.7 (http://popart.otago.ac.nz) (Bandelt et al., 1999).

Microsatellites

Microsatellite loci were one of two types of co-dominant, bi-parentally inherited markers used to test for population distinctiveness among individuals across sample locations. Samples were

genotyped using twelve newly designed polymorphic microsatellite loci, as outlined in the supplementary material (Supplementary Material and Methods, Appendix A), the methods of which included: NGS microsatellite loci detection, characterisation and optimisation of microsatellite primers (including GenBank Accession numbers) (supplementary material, Table A.1). In order to accurately size alleles, amplified products were run alongside GeneScan 500 Liz on an ABI 3130XL AutoDNA sequencer (Applied Biosystems) in the CSIRO marine laboratories. Genotypes were scored using the Microsatellite plug-in in Geneious R10.2.3 (Biomatters Ltd). To check for potential scoring errors and the presence of null alleles we used MICRO-CHECKER v2.2.3 (Van Oosterhout et al., 2004). At each locus and location we calculated the number of alleles (N_A), expected (H_E) and observed (H_O) heterozygosities, allelic richness (A_R), fixation indices (F_{IS}) and deviations from Hardy-Weinberg Equilibrium (HWE_p) using R-Package '*diveRsity*' (Keenan et al., 2013) (Table 2.1; supplementary material, Table A.2). Allele frequencies are available in supplementary material (Table A.3). To detect non-random associations of alleles among multiple loci, exact tests for linkage disequilibrium were undertaken using GENEPOP on the web v4.2 (Raymond et al., 1995).

Single Nucleotide Polymorphisms

We used a reduced-representation NGS approach to obtain SNPs from across the *C. albimarginatus* genome. We sent genomic DNA to the Australian Genome Research Facility (AGRF, www.agrf.com.au) for library preparation (including ligation of barcoded adapters, size selection of pooled digested-ligated fragments and amplification of libraries via PCR using indexed primers), and sequencing according to their in-house Genotype-by-Sequencing (GBS) methodology (Elshire et al., 2011). This is a reduced representation approach similar to ddRAD (Peterson et al., 2012), which sequences short sections of the genome selected from restriction enzyme cut-sites (enzymes PstI and MseI were used). The libraries from each of the two plates of DNA were sequenced on four lanes of an Illumina© NextSeq 500 platform flow cell (Illumina Inc, USA) with 150 cycles in MID-output mode resulting in over 410 million 100bp single end reads. AGRF processed the raw reads using their in-house STACKS pipeline v1.47, (http://catchenlab.life.illinois.edu/stacks/) (Catchen et al., 2013). The STACKS program aligns sequence reads into matching stacks from which loci are formed and SNPs are detected. The parameters used to define a 'stack' were as follows; minimum depth coverage of two (m), one mismatch allowed between sample tag (n), a minimum of five reads to call a homozygote and a minor allele frequency per stack of 0.05-1 for calling heterozygotes. All resulting SNPs were further filtered using R-Packages '*vcfR*', '*adegenet*' and '*dartR*' (Jombart et al., 2010; Knaus et al., 2017; Gruber et al., 2018) according to the following criteria: (a) only one SNP per

tag, (b) average read depth > 5, (c) no missing data per SNP, (d) minor allele frequency > 0.02, (e) no loci out of Hardy-Weinberg Equilibrium and (f) heterozygosity per individual between 0.11-0.18. This heterozygosity threshold was selected due to excessive low and high heterozygosity likely representing poor DNA quality or sample contamination respectively, (see supplementary material, Figure A.1). Step-wise filtering and SNP retention is described in supplementary material, Table A.4). Step-wise filtering and SNP retention is described in supplementary material, Table A.4). Missing data per SNP was filtered step-wise; firstly SNPs were filtered with a 30% missing data threshold, then again with no missing data threshold at the end of the filtering process (Table A.4). This was to reduce the number of SNPs in the final suite and decrease computation time. Summary statistics including, H_E and H_O, F_{IS} and A_R were calculated using R-package '*diveRsity*' (Keenan et al., 2013).

Power Analyses

In order to determine the theoretical statistical power of the microsatellite and SNP loci to resolve genetic differentiation we ran a power analysis using POWSIM v4.1 (Ryman et al., 2006). The settings of effective population size (N*e*) and generations of drift (t) were selected to represent F_{ST} values generated from pairwise comparisons identified in this study (see Ryman and Palm (2006) for F_{ST} equation). Empirical allele frequencies used in POWSIM calculations for microsatellites and SNPs were identified using R-package '*PopGenReport'* (Adamack et al., 2014). The parameters of the Markov Chain were fixed to 10,000, 1,000 and 10,000 for dememorizations, batches and iterations per run respectively. A total of 1,000 replicates of each run was completed for microsatellites and 200 for SNPs.

Population Structure

In order to test for genetic homogeneity between locations we calculated the pairwise Φ_{ST} for mtDNA and F_{ST} for the nuclear markers (Msats and SNPs) using Arelquin v3.5 and R-package '*StAMPP'* respectively (Excoffier et al., 2006; Pembleton et al., 2013). Each analysis consisted of > 10,000 bootstraps generating confidence intervals and *p*-values for each pairwise comparison. Significance levels of all pairwise tests were corrected for multiple comparisons with a sequential Bonferroni procedure (BF*p* = conventional *p*-value 0.05 divided by number of tests per marker type) (Rice, 1989).

To estimate the number of genetic groups based on the microsatellite data, we used Bayesian algorithms implemented in STRUCTURE v2.3.4 (Pritchard et al., 2000). STRUCTURE analysis

was run using an admixture model with correlated allele frequencies, a burn-in length of 50,000 followed by 1,000,000 MCMC with K (number of clusters) set between 1-7 with 8 runs for each K value. Given STRUCTURE's inability to accurately cluster individuals to populations at low levels of differentiation (Latch et al., 2006) as is the case for east Australian and PNG comparisons, a LOCPRIOR approach similar to (Falush et al., 2003) was applied with *a priori* location information. Additionally, to overcome our unbalanced sample sizes, an alternative ancestry prior of $\alpha = 0.33$ was used as suggested by (Wang, 2017).

Estimation of the number of genetic groups identified with SNP loci was undertaken using maximum likelihood algorithms in ADMIXTURE. ADMIXTURE estimates individual ancestry from SNP datasets using similar statistical models as STRUCTURE, however is computationally faster (Alexander et al., 2011). The unsupervised clustering algorithm implemented in ADMIXTURE was applied with K varying from 1- 9 with 20,000 bootstraps. A 100-fold cross-validation (CV) was set to determine the number of clusters with the lowest CV error.

We conducted an alternative assessment of genetic clusters for microsatellites and SNPs using a Discriminant Analysis of Principal Components (*DAPC)* in R-package '*adegenet'* (Jombart et al., 2010). DAPC identifies clusters by sequential clustering and model selection, this multivariate analysis does not require populations to be in HWE or linkage equilibrium (Jombart, 2008; Jombart et al., 2010). As per instructions, a third of the PCs were retained and all discriminant eigenvalues were used (< 5 for both microsatellites and SNPs).

Kinship inference

To account for potential family bias (see Feutry et al., 2017; Devloo-Delva et al., 2019), the filtered SNP data was analysed to identify kinship as described in (Hillary et al., 2018). Briefly, after allele frequencies were estimated, duplicate or replicate individuals were checked based on the number of identical genotypes. Full sibling pairs (FSPs) and parent-offspring pairs (POPs) were estimated, based on a likelihood ratio of two individuals to be either FSP/POP or unrelated (UP). At each locus, this likelihood score is calculated based on the expected probabilities that two individuals will share a genotype (according to the identity-by-descent theory, Thompson 2013) and the observed genotypes between the pair of individuals. The log-transformation of the mean of each locus-specific score compared between individuals (i.e. pseudo log likelihood or PLOD score) allows us to determine the kin relationship. MtDNA haplotypes were used to assess the proposed kin-groupings. FSP and POP were distinguished based on their cohort data. For simplicity, we only used

SNPs to infer kinship since these have proven to perform with high resolution and precision (see Hellmann et al., 2016, Attard et al., 2018).

2.3 RESULTS

By genotyping the same set of samples with a range of markers, we maximised our ability to discern population structure with available samples. The number of samples (n) successfully analysed for each marker is described in Table 2.1. Sample dropout (i.e. the loss of samples from analyses) was due to a range of factors (e.g. poor quality gDNA) which affected sequencing and genotyping success. All samples were checked for inadvertent duplication using SNPs to ensure no double sampling occurred and no duplicates were identified. Following the kinship inference using SNPs, three FSPs and three POPs were identified in our data with individuals retained in analyses. The removal of sibs (that are not a sampling artefact) can introduce more bias than their retention as suggested from empirical and simulated datasets (Waples et al., 2017). Further description of kinship results is below in kinship inference section.

Mitochondrial DNA

To investigate the relationship among mitochondrial genomes we sequenced 994bp of the mtDNA CR across 120 individuals, resulting in 14 haplotypes (GenBank accession numbers MH213460-MH213474). The majority of samples were represented by three haplotypes; two within the central Indo-Pacific and one located in the west Indo-Pacific (Figure 2.2). The number of haplotypes per location ranged from 2 (Seychelles/east Australia) to 12 (PNG); as a result, nucleotide diversities were greatest for PNG (0.144±0.10) (Table 2.1). All central Indo-Pacific haplotypes were separated from the west Indo-Pacific haplotypes by an 9bp difference (Figure 2.2).

Microsatellites

Twelve microsatellite loci were successfully genotyped in 117 individuals across the three locations. All loci were shown to be polymorphic in PNG while three loci (ALS11, ALS14, ALS51) and two loci (ALS11, ALS51) were monomorphic in Seychelles and east Australia respectively (supplementary material, Table A.2). Consequently, N_A ranged from 1 to 22, the widely variable alleles per locus was also represented in H_o values ranging from 0.00-0.900, (Table A.2). Microchecker (Van Oosterhout et

al., 2004) indicated some evidence for the presence of null alleles per location (at 2 out of 36 loci). To test the significance of these results, all loci were checked for departures from HWE. Four out of 36 tests (ALS1, ALS7, ALS42 and ALS51) were found to significantly deviate from HWE in either PNG or east Australia. Since no single locus deviated at every location, no further action was taken and all 12 loci were included in further analyses. Additionally, assessment of LD between any two loci per population found no significant association was present. Per location, loci were moderately polymorphic across all populations; average A_R = 3.63-4.70 and average H_o = 0.393-0.446 (Table 2.1).

Single Nucleotide Polymorphism

ddRAD genotyping and the STACKS pipeline returned 717,800 SNP reads. After additional stringent quality filtering (supplementary material, Table A.4), we identified a total of 6,461 SNPs polymorphic across the three locations in 92 individuals (Table 2.1). The number of polymorphic loci per population varied, with east Australia having the highest (6,014), and the Seychelles having the lowest (4,128) (Table 2.1). A_R was identical between locations (1.95) and average H_o was small and similar between locations ranging from 0.126-0.130 (Table 2.1).

Power Analysis

Power simulations suggested the 12 microsatellite loci would be sufficient to recognise population differentiation for F_{ST} values between 0.01-0.05, (power to detect > 98%), however the power quickly declined with decreasing F_{ST}, for example detecting differentiation of F_{ST} = 0.001 was calculated to be detected only 10% of the time (supplementary material, Table A.6). By contrast, the SNP dataset provided consistently high power (1) for every F_{ST} scenario tested (Table A.6).

Table 2.1. Summary of various measures of genetic diversity (averages given) for mtDNA, microsatellites and SNP datasets across the three *C. albimarginatus* sampling location.

	Seychelles (n = 31)	Papua New Guinea (n = 98)	East Australia (n = 23)
mtDNA CR (994bp)			
n	26	75	19
S	1	8	1
H	2	12	2
h	0.073	0.818	0.498
$\pi \times 10^2$	0.007±0.017	0.144±0.10	0.053±0.05
Microsatellites (12 loci)			
n	30	64	23
A_R	3.63	4.70	4.67
H_O	0.393	0.446	0.434
H_E	0.366	0.466	0.457
F_{IS}	-0.08	0.105	0.072
SNPs (6,461 loci)			
n	20	53	19
S	4128	4965	6014
A_R	1.95	1.95	1.95
H_O	0.130	0.127	0.126
H_E	0.152	0.142	0.139
F_{IS}	0.115	0.095	0.067

Table describes for each location, the number of individuals successfully amplified per marker (*n),* the observed (H_O) and expected heterozygosity (H_E), the number of polymorphic sites (S); for SNPs one site equals one locus, number of Haplotypes (H), haplotype diversity (*h*), nucleotide diversity (π), allele richness (A_R) and inbreeding coefficient (F_{IS}). Total number of individuals collected are included under the location name.

Population Structure

An assessment of fixation indices for spatial population structure identified varying differentiation between locations dependent on the marker used. Samples within PNG were tested for genetic homogeneity (based on all 3 marker types) between sample collection sites. Homogeneity could not be rejected, therefore subsequent analyses including PNG samples were considered to represent a single population. For maternally-inherited mtDNA, pairwise Φ_{ST} estimates between Seychelles and the central Indo-Pacific locations, PNG and east Australia, were very high and significantly different (Φ_{ST} = 0.920, Φ_{ST} = 0.980, $p < 0.000$ respectively) (Table 2.2). Low (albeit significant, $p < 0.000$) differentiation between PNG and east Australia was identified with Φ_{ST} value = 0.102.

The significance levels of microsatellite pairwise comparisons were similar to those of mtDNA albeit F_{ST} values were far lower ranging from 0.000 to 0.050. Again, the Seychelles was found to be significantly differentiated from central Indo-Pacific locations (PNG F_{ST} = 0.036, $p < 0.000$ and east Australia F_{ST} = 0.050, $p < 0.000$) however no genetic differentiation was identified between PNG and east Australia (F_{ST} = 0.000) (Table 2.2). Both Arlequin (which permutes genotypes between populations) and StAMPP (which bootstraps across loci) estimated pairwise F_{ST}, the F_{ST} values reported here are from Arlequin, StAMPP calculations yielded identical results (data not shown).

Estimates of population structure using nuclear SNP markers were similar to the microsatellite results. Pairwise F_{ST} values were slightly higher than for the microsatellites (SNP F_{ST} = 0.001-0.059). Significant structuring between populations was found for the Seychelles and central Indo-Pacific locations ($p < 0.000$), while again low and (after Bonferroni correction) non-significant differentiation between PNG and east Australia was identified (F_{ST} = 0.001, p = 0.017) (Table 2).

The Bayesian clustering analysis STRUCTURE was run using microsatellite loci to determine what, if any genetically similar clusters could be assigned. LnP and ΔK could not discern if K = 1 or K = 2 due to the low LnP scores between K = 1-2. Therefore, STRUCTURE results are presented for a range of K values to explore subdivision. Clustering scenario K = 2, was consistent with geographic location as STRUCTURE clearly separated individuals from the Seychelles into a cluster distinct from central Indo-Pacific individuals (Figure 2.2). Additionally, when K = 3 some individuals exclusively in the east Australia location were assigned to a separate cluster. The unsupervised clustering algorithm from the ADMIXTURE software was able to determine an optimal K using SNP markers. We identified an optimal K = 2 clusters, based on the lowest CV error (supplementary material, Figure A.3). The ADMIXTURE plot for two clusters identified more distinct separation between the Seychelles and the central Indo-Pacific locations, than the microsatellite STRUCTURE plot. Moreover,

no structure was visible between PNG and east Australia, even when increasing the number of clusters. DAPC analysis using microsatellite loci showed individuals across all three locations to occasionally overlap along the x and y-axis (Figure 2.2). Conversely, DAPC analysis for SNPs identified two clearly defined clusters consistent with geographic locations. The Seychelles individuals belonged to one cluster separated along the x-axis, while PNG and east Australia individuals made up the second cluster, closely located but slightly partitioned, along the y-axis (Figure 2.2).

Table 2.2. Global and pairwise genetic differences (Φ_{ST} and F_{ST}) calculated from 994bp mtDNA CR region, 12 microsatellite markers (unbiased G_{ST} estimate given in parenthesis) and 6,461 SNPs for *C. albimarginatus*.

		Seychelles	Papua New Guinea	East Australia
Mitochondrial DNA	Seychelles	*	0.000	0.000
Global Φ_{ST} = 0.889	Papua New Guinea	0.920	*	0.005
	East Australia	0.980	0.102	*
Microsatellites	Seychelles	*	0.000	0.000
Global F_{ST} = 0.025	Papua New Guinea	0.036 (0.018)	*	0.505
	East Australia	0.050 (0.025)	0.000 (0.000)	*
SNPs	Seychelles	*	0.000	0.000
Global F_{ST} = 0.037	Papua New Guinea	0.057	*	0.017
	East Australia	0.059	0.001	*

Above diagonal; p-values, below diagonal; pairwise Φ_{ST} and F_{ST} values; significant p values following Bonferroni correction (BFp < 0.0167) are shaded grey.

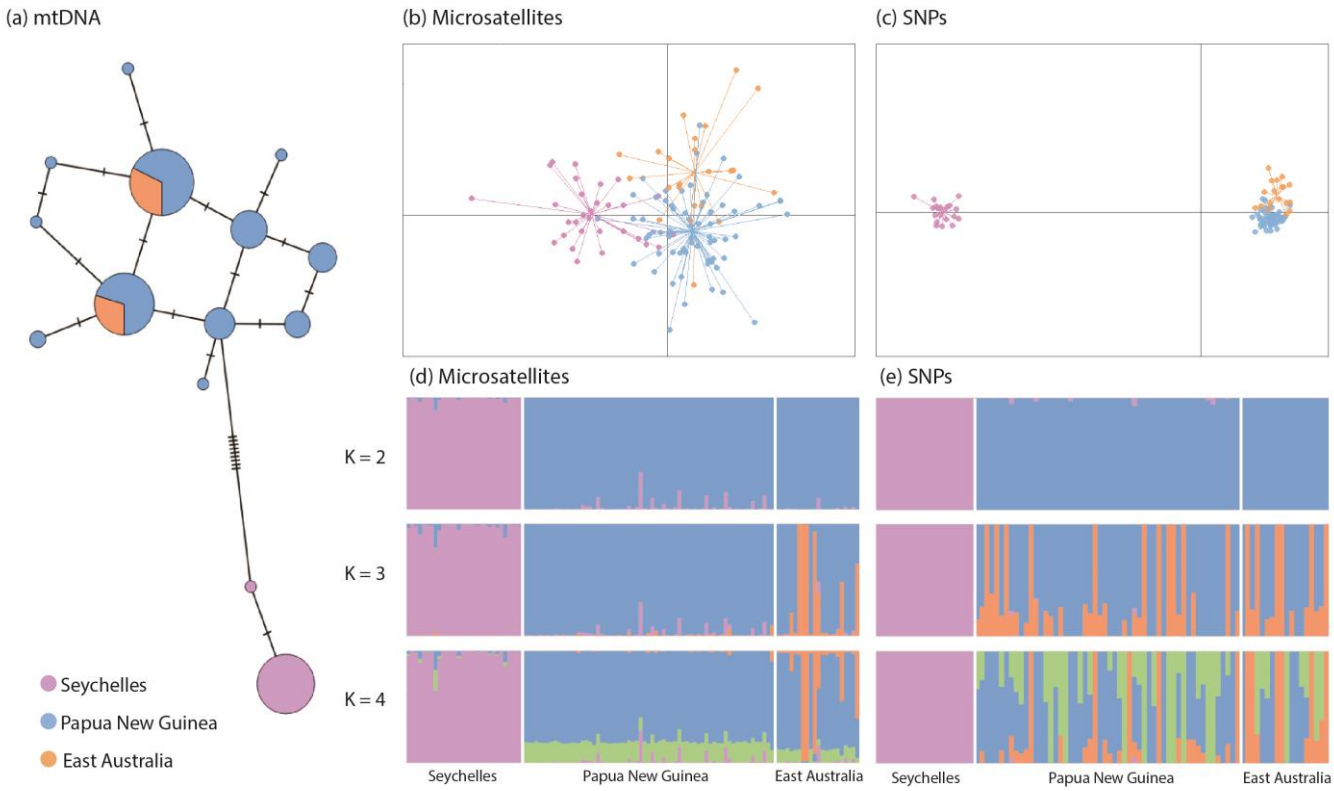

Figure 2.2. Various measurements of population structure using each marker. (a) Mitochondrial DNA (Control Region) Median-Joining network analysis from POPart v1.7. Haplotype frequencies are relative to the size of the circles, colours represent sampling locations. Number of strokes joining nodes represents number of mutations between two haplotypes (across the 994bp fragment). Scatterplot created using DAPC showing variation between individuals (dots) and populations (colours) for (b) microsatellites and (c) SNP makers. Below, corresponding cluster analyses using 12 microsatellite loci conducted in STRUCTURE (left) and 6,461 SNPs using ADMIXTURE (right). Colours represent different clusters as defined by K values.

Kinship inference

Three FSPs and three POPs were identified in our data (PLOD scores 0.024-0.181, supplementary material Figure A.4 and Table A.5). Following identification of related individuals, mtDNA haplotypes were checked for similarities. All FSP and POP individuals had matching mtDNA haplotypes with the exclusion of one POP (10177 and 10219) who had a single point mutation along the 994bp CR sequence from Thymine (T) in the mother to Cytosine (C) in the daughter. Related pairs were captured in the same locations with a maximum of 11 days between capture. Estimated age and relationship of individuals identified in analysis can be found in supplementary material (Table A.5).

2.4 DISCUSSION

Population genetic analysis of *C. albimarginatus* in the Indo-Pacific region may suggest that some level of gene flow and genetic connectivity is present between PNG and east Australia. Conversely, no connectivity was identified between the two Pacific locations and the Seychelles suggesting the Indian Ocean presents a strong barrier to gene flow between these locations. Both suites of microsatellites and SNPs were deemed powerful enough to identify population structure (as indicated in POWSIM analyses) at low levels of genetic differentiation. Our findings make important comparisons between nuclear markers providing greater confidence in our results and help describe the genetic stock structure for *C. albimarginatus* in the region.

All three marker types detected substantial genetic subdivision between individuals in the Seychelles and central Indo-Pacific locations. The lack of genetic connectivity between the two regions is consistent with our understanding that many marine taxa, in particular reef associated sharks, rarely transverse expansive ocean basins (Chapman et al., 2005; Lowe et al., 2006; Heupel et al., 2010; Whitney et al., 2012 a; Dudgeon et al., 2013; Momigliano et al., 2015; McKibben et al., n.d.). The relatively high pairwise differentiation values identified across all three markers are a strong indication that very little (or possibly no) migration is occurring across the ocean basins (i.e. resulting in the exchange of genes, where individual migrants successfully join the local population). Similar levels of population subdivision between ocean basins has been recorded for tope sharks (*Galeorhinus galeus*) (average Φ_{ST} = 0.750), spiny dog fish (*Squalus acanthias*) (Φ_{ST} = 0.744, F_{ST} = 0.055) and the scalloped hammerhead (*Sphyrna lewini*) (average Φ_{ST} = 0.499 and F_{ST} = 0.041) (Chabot et al., 2009; Veríssimo et al., 2010; Daly-Engel et al., 2012). Several other complementary lines of evidence support the low likelihood of *C. albimarginatus* individuals migrating across the Indian Ocean.

Globally, *C. albimarginatus* have a patchy and isolated distribution inhabiting exclusively coral reefs and bathymetric structures on continental shelves (Last et al., 2009). Displaying pelagic behaviours, *C. albimarginatus* primarily occupy depths between 0-60m (Espinoza et al., 2015 a), but on occasion deep dive to 400-800m (Bond et al., 2015; Espinoza et al., 2016). Coral reefs provide refuge, foraging grounds and breeding opportunities for *C. albimarginatus* (Espinoza et al., 2014 a), these essential requirements likely facilitate residency within a single ocean basin. It would be of interest to sample alternative West Indian Ocean locations including Madagascar and the east African coast to quantify levels of gene flow between these more closely located regions. Additionally, sampling from a mid-point of the species distribution across the Indian Ocean (e.g. East

Indian coast/Sri Lanka) would allow us to test whether stepping stone migrations are occurring across the ocean basin.

Genetic connectivity between PNG and the east Australia is described in a number of tests, and the low F_{ST} values (F_{ST} 0.000-0.001) in nuclear markers suggests some level of gene flow between these regions. Cluster analyses completed in ADMIXTURE and DAPC for SNPs identified genetic connectivity between PNG and east Australia consistent with microsatellite STRUCTURE results (Figure 2.2).

This study was unable to reject the null hypothesis of genetic homogeneity between PNG and east Australia for *C. albimarginatus.* If confirmed by future studies with higher sample numbers and more collection locations then connectivity would be similar to other reef-associated species within the western Pacific. Patterns of high gene flow at similar spatial scales have been reported in other reef sharks including the white tip reef shark (*Triaenodon obesus*) (Whitney et al., 2012), blacktip reef shark (*C. melanopterus*) (Vignaud et al., 2014), grey reef shark (*C. amblyrhynchos*) (Momigliano et al., 2017), *S. lewini* (Ovenden *et al.,* 2009) and tiger shark (*Galeocerdo cuvier*) (Holmes et al., 2017). More widely, factors that curtail dispersal across the Indo-Pacific include: ocean depth (Ovenden et al., 2009; Karl et al., 2012), body size (Espinoza et al., 2015a; b), temperature (Keeney et al., 2006; Verissimo et al., 2011), reproduction (Momigliano et al., 2017) and oceanographic features (Dudgeon et al., 2009). The body size of *C. albimarginatus* is larger than most pelagic reef-associated sharks suggesting its dispersal potential may be similar to that of other large bodied sharks including *S. lewini* and blue sharks (*Prionace glauca*) (Ovenden et al., 2009).

The genetic homogeneity identified between Australia and PNG does not strictly indicate that individuals are exchanging between PNG and the east coast of Australia. Certainly our identification of related pairs would suggest some level of familiar residency is occurring. On Wheeler Reef east Australia and Sudest Island PNG, mothers were captured in the same locations as their pups. Additionally a sibling pair in Manus Island PNG with an estimated age of 6-7 years were collected in the same location. All offspring and sibling pairs identified had not yet reached estimated age at maturity (Smart et al., 2017 b). Tagging studies have found *C. albimarginatus* to remain fairly resident at coral reefs, however some individuals are recorded leaving an acoustic array for a short period of time before returning; a behaviour suggested to be associated to reproduction with individuals in neighbouring reefs (Espinoza et al., 2015 a). Mating occurring between proximal individuals, leads to patterns of close relatedness at fine scales and creates genetic gradients at large scales (Schwartz et al., 2009). For example, patterns of high gene flow identified in *C. amblyrhynchos* is thought to be facilitated by nearby coral reefs representing stepping stones allowing for the

existence of genetic connectivity along the continental shelf (Momigliano et al., 2017). Telemetry work completed by Espinoza et al., (2015 a) suggested that *C. albimarginatus* individuals are dispersing to breed with individuals on neighbouring reefs. If a stepping stone style of migration is occurring this would homogenise gene flow between the two locations with subtle population structure between locations possible, however undetected by our genetic methods and sampling regime. Our results work towards supporting this hypothesis, however further robust testing using more temporally similar sampling at locations between PNG and east Australia is required to better understand the effect of geographic distance on gene flow and if any subtle population structure is apparent.

While the nuclear data is unable to reject our null hypothesis of genetic homogeneity between PNG and east Australia, the mitochondrial DNA data points to relatively low (Φ_{ST} value = 0.102) but significant population subdivision between the two sampling areas. However, this is not an unexpected finding and has been observed in other mitochondrial studies of some reef sharks. MtDNA is maternally inherited and many reef sharks show strong population subdivision between regions with discontinuous coastline (Blower et al., 2012; Daly-Engel et al., 2012; Geraghty et al., 2014; Osgood et al., 2015; Corrigan et al., 2016). Structure identified using mtDNA (and a correctly designed sampling strategy) is often linked with female-mediated residency or philopatry, whereby female sharks remain at a site or return to a natal site to give birth (Chapman et al., 2015). The low yet significant mtDNA population structure identified between PNG and east Australia may suggest female *C. albimarginatus* display residency or philopatric behaviour. While studies have found no significant difference in male and female movements, tagging was conducted for juvenile *C. albimarginatus* individuals and therefore cannot provide insight into putative philopatry (Espinoza et al., 2015 a). Kinship inference provides some qualitative evidence of philopatry, with the identification of a POP with a 16-18 year old mother and her 7-8 year old female pup present at the same location collected one day apart in Sudest Island, PNG. Further investigation of this putative behaviour is warranted including more kinship studies, tagging of mature male and female *C. albimarginatus*, removing juveniles from genetic analysis (not possible in our study due to small sample sizes) and locating nursery areas (if any) in order to understand the breeding behaviours of female *C. albimarginatus*.

Often population genetic studies show discrepancies in results between different marker types, in particular nuclear microsatellites and SNPs (Elbers et al., 2017; Vendrami et al., 2017). The concordant results from our nuclear analyses show both markers indicate similar patterns of gene flow throughout the west Indo-Pacific and central Indo-Pacific. When compared directly, one microsatellite locus contains more information than a single SNP locus, since microsatellites are

multi-allelic, and SNPs are bi-allelic (Coates et al., 2009). However, comparisons between microsatellites and SNPs and the exclusive use of genome-wide SNPs are becoming more common in population genetic literature. While small numbers of SNPs are inferior or provide similar results as microsatellites for population diversity studies (Hamblin et al., 2007; Narum et al., 2008; Coates et al., 2009; Hess et al., 2011), once the number of SNP loci increase into the thousands, their power to detect population structure based on genetic informativeness increases (Rosenberg et al., 2003; Morin et al., 2004; Rasic et al., 2014; Vendrami et al., 2017). POWSIM estimates from our study have shown the suites of 12 microsatellite and 6,461 SNP markers are both powerful enough to detect genetic population at their relative global F_{ST} values (Global F_{ST} Msats = 0.025, SNPs = 0.037). Thus the capacity of our study to test and compare the results of nuclear markers (microsatellites and SNPs), has provided a robust assessment of *C. albimarginatus* population structure.

Throughout PNG and Australia, *C. albimarginatus* are captured in commercial, small-scale and IUU fisheries (Kumoru, 2003; Marshall, 2011; Bond et al., 2015; Smart et al., 2017 b). Their susceptibility as by-catch during fishing has led to their Vulnerable status under the IUCN Red List (Espinoza et al., 2016), with *C. albimarginatus* at risk of declining populations as they lack the capacity to be harvested sustainably unless fishing is limited to specific age-classes (Smart et al., 2017 a). Like many other shark species, *C. albimarginatus* have low fecundity and long generation times, with demographic modelling finding populations unable to tolerate moderate levels of harvesting when all age classes are fished (Smart et al., 2017 a). It has been demonstrated that low levels of by-catch of young of the year (YOY) up to a maximum size of 100cm (TL) is the most sustainable option for *C. albimarginatus* (Smart et al., 2017 a). Contrastingly, samples collected during this project from the PNG region were harvested in longline fisheries and all individuals, except one, were over 100cm TL. Furthermore, *C. albimarginatus* individuals collected from artisanal fisheries in PNG have recently been estimated at catch sizes between 68-201cm TL for 28 individuals (Appleyard et al., 2018). In northern Australia, IUU fishing has reported a high proportion of adults being targeted (Marshall 2011). Concerningly, populations of *C. albimarginatus* in PNG and Australia will unlikely be able to recover if the breeding adults are persistently removed.

Our study has suggested genetic connectivity apparent between east coast Australia and Papua New Guinea, each of which has varying degrees of fishing pressure and fisheries management capabilities for this species. Given these results we suggest the regions are considered and managed as a single genetic stock. We also recommend that both nations consider reducing catches of *C. albimarginatus* over 100cm (TL), possibly by changing gear type and or harvesting locations, in order to protect the adult population and avoid recruitment overfishing.

Conclusion

The lack of genetic connectivity between the Seychelles and the two central Indo-Pacific locations, shown here for both mitochondrial and nuclear DNA was not unexpected due to the large geographic distance over ocean basins. However, potential genetic connectivity between PNG and east Australia is of great interest. Whether the identified connectivity is an effect of stepping stone migrations creating a genetic gradient or indicative of direct exchange of individuals between locations cannot be resolved with our available data. Additionally, mtDNA differentiation between PNG and Australia might perhaps reflect assumed female philopatry. Our suggestion of genetic connectivity between PNG and east Australia provides important evidence that *C. albimarginatus* may have large home ranges within which movement and mating is extensive.

The use of multiple markers in this study provided a robust comparison and furthermore adds to the growing literature describing genetic population structure for elasmobranchs based on multiple approaches. Our research highlights the benefits of combining multiple lines of evidence with previously available tagging information to better understand movements of large-bodied marine species, the output of which can provide information to fisheries managers in the region.

Chapter 3 Genetic connectivity of the scalloped hammerhead (*Sphyrna lewini*) in the Pacific and Indian Oceans using a multi-marker approach

3.1 INTRODUCTION

Knowledge of the biological stock structure of highly mobile marine species provides a basis for informed management for fisheries or conservation commitments. The identification of biological stock structure is challenging given many broad ranging species lack obvious barriers to dispersal (Cowen et al., 2006). Until formally tested, it is often assumed a species belongs to a single panmictic stock, leading to complex and challenging international management requirements (Ward, 2000; Reiss et al., 2009; Chin et al., 2017). Despite a relative lack of physical barriers preventing shark dispersal, we often see biological stock structure driven by more subtle environmental barriers relating to an individual's requirements of habitat, food and reproduction. For large bodied sharks that are subject to high harvest pressure, biological stocks are often found to occur across Exclusive Economic Zones (EEZs) of a number of countries, including International waters, requiring cross-jurisdictional consultation and management (Ovenden et al., 2015; Chin et al., 2017; Vaudo et al., 2017).

The scalloped hammerhead (*Sphyrna lewini*) is one of ten currently recognised hammerhead shark species. It is a large bodied shark with a circumglobal distribution in tropical and warm-temperate waters (Last et al., 2009). Adults are often found occupying oceanic seamounts and continental shelves in depths of more than 275m, with reports of aggregation and long distance dispersive behaviours (Klimley et al., 1981; Compagno et al., 2005; Hearn et al., 2010; Bessudo et al., 2011). There are a number of studies describing the seasonal migrations of adult females into sheltered coastal waters to give birth (Clarke, 1971; Bessudo et al., 2011; Yates et al., 2015) and it has been suggested that the species experience female-mediated philopatry (Daly-Engel et al., 2012). Multiple mating events in a single season are possible for *S. lewini*, leading to litters containing pups sired by multiple fathers (Green et al., 2017). Young-of-the-year (YOY), neonates and juvenile *S. lewini* remain in shallow coastal areas in depths of less than 100m, likely increasing the chance of protection from large predators (Heupel et al., 2007, 2018 a). A number of pupping grounds have been identified for *S. lewini* including in the Gulf of California (Baum et al., 2007), the inshore regions of the east coast of Australia (i.e. Cleaveland, Bowling Green, Upstart, Edgecumbe and Repulse Bays) (Simpfendorfer et al., 1993; Yates et al., 2015), Fiji (Brown et al., 2016), and

Galapagos (Hearn et al., 2010). Ontogenetic and sex-biased shifts in habitat use have been described for *S. lewini* within the central Indo-Pacific; whereby the majority of northern Australian waters contain male neonates and juveniles while a high proportion of adult females are found in Indonesia (Chin et al., 2017).

Listed as endangered under the International Union for the Conservation of Nature (IUCN) Red List, all life stages of *S. lewini* are vulnerable to capture with a variety of fishing methods near and off shore. As such, *S. lewini* is listed as a conservation concern with many international and national bans and, restrictions established including: Appendix II listing within the Conservation on International Trade in Endangered Species (CITES, 2014), Appendix II for the Conservation of Migratory Species (CMS, 2015), listing in the U.S. Endangered Species Act (79 FR 38213) (NOAA, 2014) and listed as Endangered under the New South Wales (Australia) Fisheries Management Act (1994). Fishing is considered the major threat for *S. lewini* with methods such as trawling, purse-seining, gillnetting, longlining (bottom and pelagic) and inshore artisanal fishing (Baum et al., 2007). The fins of *S. lewini* are highly valuable due to their high fin-ray count and estimates have *S. lewini* and the smooth hammerhead (*S. zygaena*) attributing 4-5% of all shark fin in the Hong Kong fin trade (Clarke et al., 2006). As with many elasmobranchs, their slow life history characteristics (low fecundity, low growth rate, late maturation) in combination with their species-specific preference to aggregate for mating means *S. lewini* are highly susceptible to fishing pressure and are less likely to replenish population losses at a sufficient rate (Barker et al., 2005). In the Western Indian Ocean, reports of population size using fisheries independent surveys (shark control nets) found a trend of population decline across a 25 year period (1978-2003) (Dudley et al., 2006). Catch data from South Africa, northwest and western central Atlantic and Brazil has also reported significant population declines between 50-90% over 32 years (Baum et al., 2007). Total catches throughout central Indo-Pacific locations; Indonesia, Papua New Guinea (PNG) and Australia are unknown, however given the high number of elasmobranch catches reported for Indonesia the species is estimated to be at risk of being overfished in at least Indonesia waters (White et al., 2008). With available data, regional IUCN assessments found the West Indian Ocean and Eastern Central Pacific populations to be considered regionally endangered (Baum et al., 2007). Not enough information is available in Australia and consequently the regional listing is Data Deficient and the IUCN has called for urgent population assessment (Baum et al 2007).

Genetic methods have proven to deliver important assessments of the biological stock structure for the globally distributed *S. lewini* (Duncan et al., 2006 b; Ovenden et al., 2009; Daly-Engel et al., 2012). By comparing bi-parentally inherited nuclear DNA and maternally inherited mitochondrial DNA (mtDNA), female mediated gene flow is observed to be restricted to oceanic

basins and along continental shelves (Duncan et al., 2006 b; Ovenden et al., 2009; Daly-Engel et al., 2012). This finding has concluded, female residency or female philopatry is likely for the species. Conversely, studies using nuclear DNA in the form of microsatellites (hereafter referred to as 'Msats') have described genetically homogeneity for *S. lewini* across a broad region encompassing Pacific and Indian Oceans likely driven by male biased dispersal (Daly-Engel et al., 2012). Similarly, the blue shark (*Prionace glauca*) was assessed using Msats, with populations found to share genetic information spanning ocean basins (Bailleul et al., 2018). While dispersal across large oceanic basins may be possible for large bodied sharks, Bailleul et al., suggest their nuclear Msat markers may not have enough power to identify demographic isolation of biological stocks. Genetic connectivity is facilitated through the effective migration of individuals between populations independent of the population size (Wright, 1969), however demographic connectivity is reached when a proportion (10% or above) of the population are migrating (Waples et al., 2006; Lowe et al., 2010; Ovenden, 2013). Therefore, the smallest migration rates and successful reproduction (i.e. effective migration) can create genetic connectivity, however populations may remain demographically separate (Wright, 1951; Waples et al., 2006). To discern signal from noise, measuring connectivity with enough power (i.e. having large sets of variable loci and large sample sizes) is key.

Single Nucleotide Polymorphisms (SNPs) are single base-pair mutations which occur across an individual's genome, they are abundant and with increasing advances in Next-Generation sequencing technology, are easily obtainable. Accessible in the thousands, SNPs are increasingly becoming a common tool for population genetic assessments of species (Hohenlohe et al., 2018). SNPs differ from Msats as they are bi-allelic while Msats are multi-allelic. This difference in alleles available per locus means in a one-to-one comparison Msat loci are more powerful than SNP loci, however once the number of SNP loci increases into thousands, SNPs have been found to be powerful enough to detect small genetic differences (Willing et al., 2012; Malenfant et al., 2015; Jeffries et al., 2016; Momigliano et al., 2017). A number of marine species, once thought to be panmictic over broad spatial scales have now been shown to have genetic population subdivision using SNP markers (Lamichhaney et al., 2012; Benestan et al., 2015; Bradbury et al., 2015; Jeffries et al., 2016). Following the appropriate evaluation both across genetic marker types and by comparison with non-genetic sources of information (telemetry, parasites etc.), those patterns thought to reflect biological reality will result in stock delineation and robust management.

Using a species whose global genetic stock structure is well documented, this study compares and contrasts biological stock assignment for *S. lewini* using three different types of genetic markers; mtDNA, Msats and SNPs. By deploying multiple genetic markers on a well-studied species, this study has the capacity to estimate what if any differences in genetic population

structure are observed when using nuclear Msat and SNP markers. The results of which can be used as a case study to aid future marker selection for measuring population structure in elasmobranchs. Given the global endangered status of *S. lewini* and its 'Data Deficient' listing within the Oceania region, the current research outlines the most comprehensive population genetic study for any shark species to-date in order to inform the appropriate setting of conservation and management measures. This work has accessed samples from previously published work on *S. lewini* from the Pacific and Indian Oceans including; Duncan et al., (2006 b), Daly-Engel et al., (2012), Ovenden et al., (2009) and newly collected samples to test whether differences in biological stock structure are identified using a multi-marker approach.

3.2 METHODS

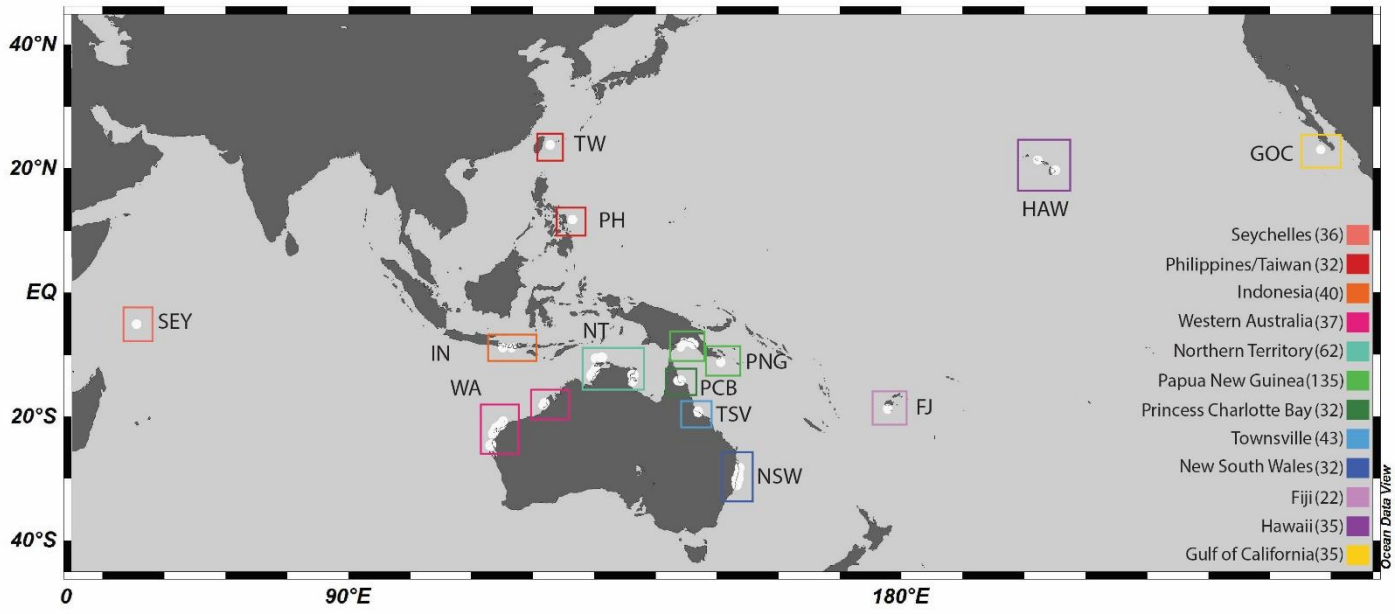

Figure 3.1. Sample collections for *S. lewini* within the Indian and Pacific Oceans. Colour squares represent location of sample collection, white dots represent sample collection sites, numbers in brackets indicate total sample size (for sample size per marker type see table 1).

Sample Collection and DNA extraction.

A total of 541 *S. lewini* DNA samples were obtained from 12 locations across the Pacific and Indian Oceans (Figure 3.1).In order to compare our multi marker study with that of previous *S. lewini* genetic assessments we accessed samples used in Daly-Engel et al., (2012) and Ovenden et al., (2009). Therefore, across all locations, collection occurred between 1999 and 2016. Collection

protocols for samples from the Seychelles (SEY), Philippines (PH), Taiwan (TW), Hawaii (HAW) and Gulf of California (GOC) are described in (Daly-Engel et al., 2012), while sampling of individuals from IN, WA and PCB is outlined in (Ovenden et al., 2009). Samples from PNG were collected on-board fishing vessels by fisheries observers and from coastal fisheries during dedicated surveys as part of an Australian Centre for International Agricultural Research (ACIAR) project (project number #FIS/2012/102). For sharks landed by commercial and coastal fishers a piece of vertebrae chord or muscle was collected. DNA was extracted from all samples using the Wizard© SV Genomic DNA Purification system (Promega, Australia); tissue extractions were undertaken using SV minicolumns following modifications to the manufacturer's instructions (i.e. overnight tissue digestion; reduced amount of supernatant to elute DNA; increased DNA elution times). Total genomic DNA (gDNA) was eluted in DNAse free water and quantified (ng/ul) on a Nanodrop 8000 (Thermo Fisher Scientific, Australia) with A260:A280 ratios reflecting DNA quality.

Mitochondrial DNA

To measure the matrilineal genetic similarity between samples from various locations we amplified two portions of mtDNA. Two markers were used to capture sufficient mtDNA SNPs to distinguish between putative genetically similar and distance populations. A 964bp of the control region (CR) and 853bp of NADH dehydrogenase subunit 4 (ND4) were amplified resulting in a total concatenated sequence of 1,817bp in length. For the CR portion forward and reverse primers PRoL2 and PheCacaH2 were used (Pardini et al., 2001), while the forward and reverse primers; ND4 and Leu-Scyliorhinus were used for ND4 amplification (Naylor et al., 2005). For both mtDNA regions Polymerase Chain Reactions (PCR) were conducted in 25 μL reactions with 15-25 ng of gDNA, GoTaq® Green Master Mix (Promega, USA), 1 μL Bovine Serum Albumin (Promega) and 10 μM primers. PCR used the following thermocycler parameters (for both CR and ND4); initial hold at 94°C/ 5 min, 35 cycles of 94°C/ 30 sec, 52°C/ 30 sec, 72°C/ 1 min, followed by final extension of 72°C/ 10 min. After PCR products were cleaned with Agencourt AMPure (Beckman Coulter, Australia), successfully amplified PCR products were sequenced bi-directionally for CR and unidirectional (forward) for ND4 using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Invitrogen Life Technologies, USA) and an annealing stage of 52°C/5 sec for 25 cycles. Cycled sequence products were cleaned using CleanSEQ kit (Beckman Coulter, Australia) and ran on an ABI 3130XL AutoDNA sequencer (Applied Biosystems, USA) at the CSIRO marine laboratories, Hobart, Australia. Sequences were screened and aligned using Geneious v10.2.3 (Biomatters Ltd, New Zealand). In order to find the best-fit substitutional model for mtDNA sequences MEGA v5.2 was used (Tamura et al., 2011).

We calculated molecular diversity indices such as haplotype and nucleotide diversities using Arlequin v3.5 (Excoffier & Lischer, 2010). To visualize haplotype networks, median-Joining network analysis was constructed using POPart v1.7 (http://popart.otago.ac.nz) (Bandelt et al., 1999). Estimates of genetic differentiation between locations was calculated using pairwise Φ_{ST} for mtDNA in Arelquin v3.5 (Excoffier & Lischer, 2010). Each analysis consisted of 10,000 bootstraps generating confidence intervals and *p* values for each pairwise comparison. Significance of pairwise tests for mtDNA and nuclear markers was considered when $p = < 0.01$ and $p = < 0.001$ respectively, in order to be comparable with Daly-Engel et al., (2012) and Ovenden et al., (2009).

Microsatellites

Microsatellite loci were one of two types of nuclear markers used to test for population distinctiveness among individuals across sample locations. Samples were genotyped using nine polymorphic Msat loci initially described in Nance et al., 2009). PCR amplifications were performed in three multiplex reactions and forward primers were labelled with a single proprietary fluorophore dye; 6-FAM, VIC, NED, PET (Applied Biosystems, USA). PCR conditions consisted of 1X GoTaq© Colourless Master Mix (Promega), 1 μL Bovine Serum Albumin (Promega,), 0.2 μM of each individual F and R primer, and 0.8 ng/µl DNA in a 25 μL reaction volume. Thermal cycling (in an Eppendorf Mastercycler®, Eppendorf, Germany) consisted of initial denaturation at 94°C/3 min, 35 cycles of 94°C/1 min, T_a of 58°C/30 sec, 72°C/1 min and a final extension of 72°C/10 min. Amplification success was visualised on agarose gels containing SYBR Safe DNA gel stain (ThermoFisher Scientific, USA). Following PCR amplifications in each of the *S. lewini* individuals, GeneScan™ 500 LIZ ™ size standard (Thermofisher Scientific) and formamide were added to 3 μL of each PCR reaction and 20 μL sample volumes were run on an ABI 3130XL AutoDNA sequencer (Thermofisher).

Genotypes were scored using the Microsatellite plug-in program in Geneious R10.2.3 (Biomatters Ltd). To check for potential scoring errors and the presence of null alleles we used MICRO-CHECKER v2.2.3 (Van Oosterhout et al., 2004). At each locus and each location we calculated the number of alleles (N_A), expected (H_E) and observed (H_O) heterozygosities, allelic richness (A_R), fixation indices (F_{IS}) and deviations from Hardy-Weinberg Equilibrium (HWE_p) using R-Package '*diveRsity*' (Keenan et al., 2013). Allele frequencies are available in supplementary material (Table B.1). To detect non-random associations of alleles among pairs of loci, exact tests for linkage disequilibrium were undertaken using GENEPOP on the web v4.2 (Raymond et al., 1995). Genetic differentiation between locations was calculated with pairwise F_{ST} for Msats using the R-package '*diveRsity'* (Keenan et al., 2013). Each analysis consisted of 100,000 bootstraps generating 95%

confidence intervals for each pairwise comparison. The number of genetic groups in our Msat dataset was estimated using Bayesian clustering algorithms implemented in STRUCTURE v2.3.4 (Pritchard et al., 2000). STRUCTURE analysis was initially run using an admixture models with correlated allele frequencies, a burn-in length of 50,000, followed by 1,000,000 MCMC with K (number of clusters) set between 1-15 and 8 iterations per value. Optimum K was determined by LnP and ΔK outputs using Structure Harvester v.0.6.94 (Evanno et al., 2005; Earl et al., 2012). We also conducted an alternative assessment of genetic clusters for Msats using a Discriminant Analysis of Principle Components (DAPC*)* in R-package '*adegenet'* (Jombart et al., 2010). DAPC identifies clusters by sequential clustering and model selection, the multivariate analyses does not require populations to be in HWE or linkage equilibrium (Jombart, 2008, Jombart et al., 2010) As per instructions from Jombart et al., (2010), the number of Principle Components (PC) retained were selected by dividing the number of individuals by three (PC = 115) and 10 eigenvalues were used.

The relationship between genetic and geographic distance was explored using Mantel tests, where the null hypothesis is that genetic difference is not correlated with geographical distance (Mantel, 1967). Analyses were undertaken following Slatkin (1995) in Arlequin v3.5 (Excoffier et al., 2005), whereby genetic distance was calculated as $F_{ST}/(1-F_{ST})$ (or $\Phi_{ST}/(1-\Phi_{ST})$ for mtDNA) and geographic distance was measured as a straight distance between locations. For nuclear markers two spatial population structure scenarios were examined on a broad scale (all locations) and at a finer scale (central Indo-Pacific locations). Central Indo-Pacific locations included PNG, PHTW, IN, WA, NT, PCB, TSV and NSW were tested separately due to belonging to a single ocean basin.

Single Nucleotide Polymorphisms

We used a reduced-representation NGS approach to obtain SNPs from across the genome. This enabled us to target and capture a subset of orthologous regions across the genome for many samples. We sent genomic DNA to the Diversities Arrays Technology Pty. Ltd (Canberra, Australia) for library preparation and sequencing using the standard DArTSeq Protocol. DArTSeq is a genotype-by-sequencing approach that uses Diversity Arrays (DArT) restriction enzymes (Jaccoud, 2001) and next-generation sequencing on an Illumina platform (Sansaloni et al., 2011). All resulting SNPs were filtered using R-Packages '*adegenet*' and '*dartR'* (Jombart et al., 2010; Gruber et al., 2018) according to criteria described in the supplementary materials (Table B.2). Broad and fine scale population structure was examined using two different suites of SNPs filtered separately. Broad scale gene flow was assessed using all locations and results in a final SNP set of 5,689 SNPs, while fine scale gene flow included central Indo-Pacific locations (PNG, PHTW, IN, WA, NT, PCB, TSV and NSW) and a set of

5,969 SNPs. Filtering of each set of SNPs is described in supplementary materials (Table B.2). Summary statistics including, H_E and H_O, F_{IS} and A_R were calculated using R-package '*diveRsity*' (Keenan et al., 2013). To describe genetic differentiation between locations we calculated pairwise F_{ST} using the R-package '*StAMPP'* (Pembleton et al., 2013)(Excoffier et al., 2006; Pembleton et al., 2013) . Each analysis consisted of > 10,000 bootstraps generating confidence intervals and *p* values for each pairwise comparison. To investigate the ancestry of each individual and number of genetic groups in our SNP datasets we used the unsupervised maximum likelihood algorithm implemented in ADMIXTURE with K varying from one to 14 and 10,000 bootstraps (Alexander et al., 2011). A 100-fold cross-validation (CV) was set to determine the optimal number of clusters for successfully reassigning individuals to their original group (i.e. lowest CV error). Alternative clustering assessment for SNPs was undertaken using DAPC in the R-package '*adegenet*' (Jombart et al., 2010). One hundred and three PCs (a third of the number of individuals in the dataset) were retained and 10 discriminant eigenvalues were used.

Genetic distance in relation to geographic distance was measured for SNPs using the *gl.ibd* function in R-package '*dartR'* (Gruber et al., 2018). Similar to Arlequin, *'dartR'* estimates isolation by distance (IBD) based on mantel tests where genetic distance is calculated as $F_{ST}/(1-F_{ST})$. Geographic distance is represented as the log of distance in meters. Two differently filtered datasets consisting of various combinations of locations were used to understand the effect of large and fine scale structure on IBD analysis (See supplementary materials for description of SNP sets).

Power Analyses

The statistical power of the Msat and SNP loci to resolve genetic differentiation was assessed with a power analysis using POWSIM v4.1 (Ryman & Palm 2006). The settings of effective population size (N*e*) and generations of drift (t) were selected to represent a range of F_{ST} values generated from pairwise comparisons identified in this study (see Ryman & Palm 2006 for F_{ST} equation). Empirical allele frequencies used in POWSIM calculations for Msats and SNPs were identified using R-package '*PopGenReport*' (Adamack et al., 2014). The parameters of the Markov Chain were fixed to 10,000, 1,000 and 10,000 for dememorizations, batches and iterations per run respectively. A total of 1,000 and 200 replicates of each run was completed for Msats and SNPs, respectively.

Table 3.1. Summary of various measures of genetic diversity (averages given) for mitochondrial DNA, microsatellites and SNP datasets in *S. lewini* across the twelve sampling locations.

			mtDNA (CR+ND4, 1817bp)					Microsatellites (9 loci)						SNPs (5689 loci)					
Ocean	Site	Abbr.	*n*	S	H	*h*	$\pi \times 10^2$	*n*	A_R	H_O	H_E	F_{IS}	HWE_p	*n*	S	A_R	H_O	H_E	F_{IS}
Indian	Seychelles	SEY	22	4	7	0.653	0.055	26	9.28	0.768	0.801	0.045	0.516	14	3,753	1.91	0.128	0.167	0.177
	Indonesia	IN	35	75	18	0.908	0.675	23	8.65	0.614	0.715	0.165	0.248	23	4,507	1.91	0.121	0.161	0.214
	West Australia	WA	10	17	6	0.780	0.478	27	9.13	0.612	0.761	0.220	0.173	21	4,491	1.92	0.130	0.166	0.178
Pacific	Philippines/Taiwan	PHTW	19	75	10	0.776	1.419	29	8.86	0.697	0.784	0.125	0.237	21	4,421	1.92	0.130	0.165	0.177
	North Australia	NT	54	27	20	0.882	0.233	33	9.13	0.743	0.783	0.048	0.204	28	4,812	1.92	0.126	0.165	0.206
	Papua New Guinea	PNG	77	82	33	0.912	0.267	37	9.57	0.746	0.800	0.073	0.416	67	5,315	1.93	0.126	0.166	0.228
	Princess Charlotte Bay	PCB	25	24	12	0.838	0.167	29	9.15	0.717	0.783	0.093	0.406	17	4,223	1.91	0.123	0.163	0.189
	Townsville	TSV	39	29	25	0.934	0.165	43	8.61	0.686	0.785	0.134	0.241	33	4,916	1.92	0.129	0.167	0.202
	New South Wales	NSW	25	23	13	0.774	0.135	30	9.09	0.738	0.788	0.076	0.311	26	4,686	1.92	0.126	0.165	0.196
	Fiji	FJ	21	5	7	0.712	0.064	22	9.43	0.770	0.792	0.027	0.383	19	4,263	1.92	0.126	0.165	0.192
	Hawaii	HAW	14	5	3	0.439	0.055	28	8.05	0.787	0.758	0.040	0.502	25	4,351	1.93	0.127	0.169	0.215
	Gulf of California	GoC	18	2	3	0.537	0.033	27	6.99	0.673	0.709	0.048	0.370	16	3,236	1.91	0.116	0.162	0.227
	Total *n*		359					354						310					

The number of individuals successfully amplified per marker and total (*n*), the observed (H_O) and expected heterozygosity (H_E), the number of polymorphic sites (S); for SNPs one site equals one locus, number of Haplotypes (H), haplotype diversity (*h*), nucleotide diversity (π), allelic richness (A_R), inbreeding coefficient (F_{IS}) and Hardy-Weinberg significance value (HWE_p).

3.3 RESULTS

The number of samples successfully analysed for each marker type is described in Table 3.1. Differences in sample size per location and per marker are likely due to a number of factors affecting sequencing and genotype success (i.e. poor quality gDNA). Due to reduced sample sizes within Philippines and Taiwan the individuals from these sampling locations were grouped together to allow the representation of the locations in the analyses. Separate pairwise analysis was conducted to ensure no difference between locations (hereafter abbreviated PHTW) occurred, since no significance was identified, samples were retained (data not shown).

Mitochondrial DNA

To describe the relationship among mitochondrial genomes of individual *S. lewini* we sequenced a 1,817bp concatenated portion of CR and ND4 regions. Maximum likelihood fits of nucleotide substitutions models found the Tamura model to have lowest BIC values for both CR and ND4 portions and was therefore used in further testing of the concatenated sequences. A total of 359 individuals from 12 populations were successfully amplified at both mtDNA regions resulting in 43 haplotypes. A large break between haplotypes of 19 mutations separated all individuals from SEY and some individuals from IN, PHTW and PNG (Figure 3.2). Upon further investigation these haplotypes were identified as very similar to those of the previously described CR 'Atlantic Ocean' haplotype of *S.lewini* (Quattro et al., 2006, 2013) (Supplementary Table B.3). Overall, the majority of other haplotypes were found to be shared in individuals from different locations. The number of haplotypes varied greatly from H = 33 in PNG to H = 3 in Hawaii and the GOC, subsequently nucleotide diversity greatly varied from $\pi = 0.014$ in PHTW to $\pi = 0.003$ in GOC (Table 3.1).

An assessment of the fixation indices for spatial population structure across the 12 populations, found a moderate and significant global Φ_{ST} of 0.622 ($p < 0.010$). Pairwise Φ_{ST} estimates for the western most location (SEY) and the eastern most location (GOC) were very high and significantly different across all locations (Φ_{ST} = 0.265-0.988, $p < 0.010$) (Table 3.2). HAW and WA were also found to be genetically dissimilar from east Australia and Fiji locations (PCB, TSV, NSW & FJ) (Φ_{ST} = 0.193-0.379, $p < 0.010$).

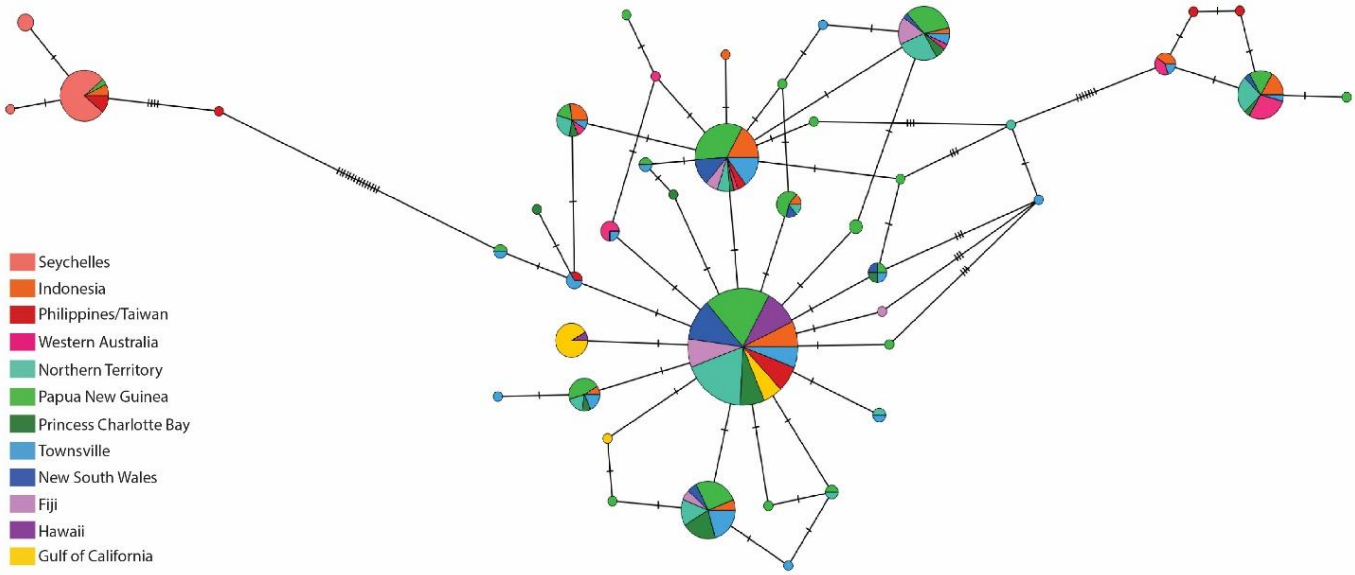

Figure 3.2. Mitochondrial DNA (CR and ND4) Median-Joining network analysis from POPart v1.7. *S. lewini* haplotype frequencies are relative to the size of the circles, colours represent sampling locations. Number of strokes joining nodes represents number of mutations between two haplotypes (across the concatenated 1817bp fragment).

Table 3.2. Pairwise genetic differences (Φ_{ST}) calculated from concatenated 1,817bp mtDNA CR and ND4 for *S. lewini*.

	SEY	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ	HAW	GOC
SEY	*	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
PHTW	0.764	*	0.140	0.118	**0.002**	**0.001**	**0.009**	**0.001**	**0.008**	**0.002**	**0.027**	**0.000**
IN	0.877	0.048	*	0.181	0.086	0.039	0.042	0.011	0.041	0.032	0.020	**0.000**
WA	0.951	0.099	0.018	*	0.014	0.003	**0.001**	**0.001**	**0.001**	**0.001**	**0.000**	**0.000**
NT	0.951	0.207	0.025	0.177	*	0.377	0.164	0.169	0.249	0.186	**0.008**	**0.000**
PNG	0.941	0.215	0.037	0.220	0.000	*	0.406	0.777	0.564	0.644	**0.002**	**0.000**
PCB	0.969	0.157	0.045	0.291	0.018	0.002	*	0.740	0.677	0.251	**0.000**	**0.000**
TSV	0.966	0.203	0.054	0.309	0.013	0.000	0.000	*	0.748	0.577	**0.000**	**0.000**
NSW	0.974	0.166	0.045	0.307	0.007	0.000	0.000	0.000	*	0.659	**0.000**	**0.000**
FJ	0.984	0.171	0.057	0.379	0.020	0.000	0.009	0.000	0.000	*	**0.000**	**0.000**
HAW	0.985	0.158	0.111	0.418	0.171	0.133	0.193	0.199	0.209	0.362	*	**0.000**
GOC	0.988	0.265	0.290	0.598	0.498	0.442	0.582	0.593	0.639	0.788	0.805	*

Above diagonal; *p* values, below diagonal; pairwise Φ_{ST} values, significant values (*p* = < 0.010) are in bold.

Microsatellites

Microsatellite loci were one of two markers used to estimate the variability in the nuclear genome. A total of 354 individuals from twelve locations successfully amplified at nine polymorphic loci. Each loci was highly variable as indicated with A_R ranging from 6.99-9.43 and H_O 0.612-0.787 (Table 3.1). All loci were checked for departures from HWE, after Bonferroni correction of *p* values (BF*p* = 0.05/108), 12 tests were considered out of HWE within some populations (Supplementary Table B.4). No single loci was out of HWE at each of the 12 locations, however WA did have the highest number of departures for a single location (3 loci with $HWE_p < 0.002$). Per loci (across all locations), no significant departures from HWE were observed, therefore all loci were retained in analyses. Assessment of LD between any two loci per population found no significant association was present. Power simulations were undertaken to ensure the suite of Msat loci were powerful enough to accurately detect F_{ST} values. The POWSIM results found nine Msat loci were sufficient to recognise population differentiation under tested scenarios for F_{ST} values between 0.001-0.05 (power to detect = 1) (Supplementary table B.5).

The global F_{ST} estimated using Msats was quite small (F_{ST} = 0.010). Pairwise F_{ST} comparisons showed the westernmost location SEY and easternmost location HAW and GOC to have the highest pairwise F_{ST} values (F_{ST} = 0.010 – 0.064), with many of the pairwise comparisons found to be highly significant (*p* = < 0.001) (Figure 3.3, Table B.6). Evidence of high gene flow as reflected in low and non-significant F_{ST} values was apparent between closely located regions within the central Indo-Pacific (PHTW, IN, WA, NT, PNG, PCB, TSV, NSW) (F_{ST} = 0.000-0.006). DAPC analysis identified some overlap between all locations, however GOC, WA, SEY and IN were more separated than all other locations (Figure 3.4).The Bayesian clustering analysis STRUCTURE was run using Msats to determine what, if any genetically similar clusters could be assigned (Figure 3.6). Based on the Evanno method K = 3 was the most likely clustering using the Msat dataset (Figure B.1). In all plotted scenarios (Figure 3.6, K = 2-6) GOC was separated into its own distinct cluster, while almost all the other locations were similarly assigned to other clusters. Genetic and geographic distances were found to correlate when all populations were included in the analysis (Figure 3.5). The all location dataset was found to be significantly correlated with r values of 0.74 (*p* = < 0.005). No relationship between genetic and geographic distance could be identified between central Indo-Pacific locations.

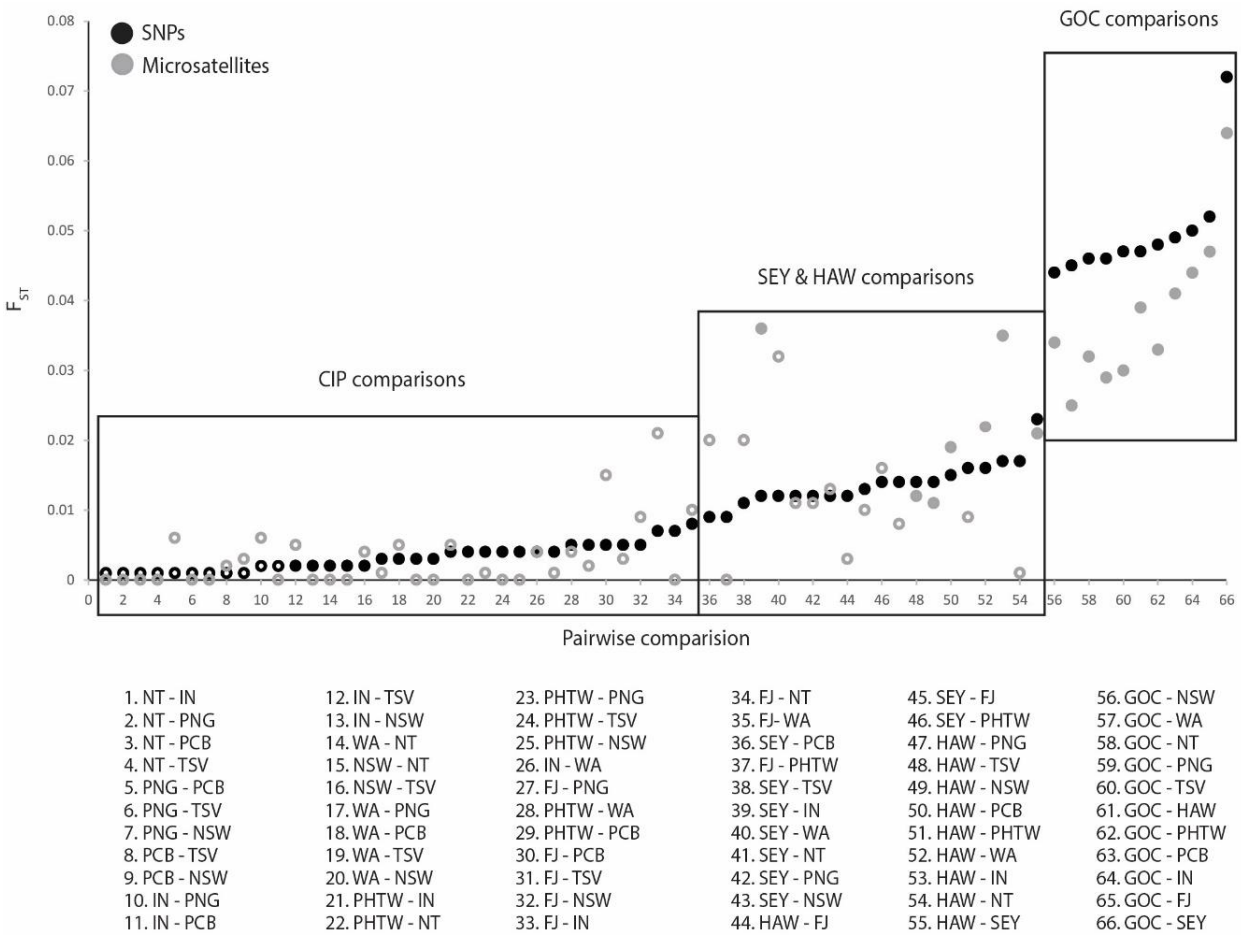

Figure 3.3. Estimates of pairwise genetic differentiation (F_{ST}) between all sampled locations for *S. lewini* using SNP (black) and microsatellite (grey) loci. Where CIP = central Indo-Pacific, SEY = Seychelles, HAW = Hawaii and GOC = Gulf of California. Comparisons are arranged in ascending order of SNP F_{ST} values (x-axis). Filled circles indicate significant *p*-values where $p = < 0.001$ and boxes represent pairwise comparisons between grouped locations (note 37 is the only CIP comparison within the SEY & HAW section).

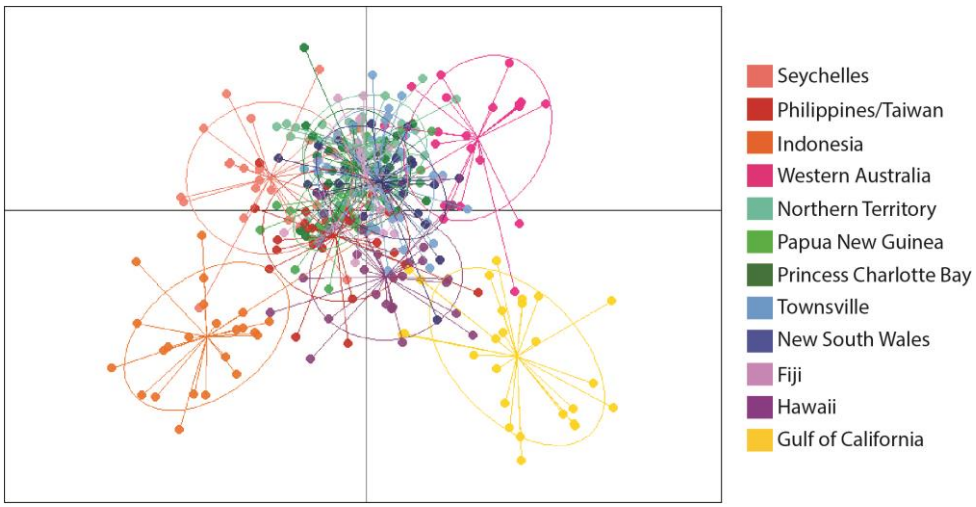

Figure 3.4. Scatterplot created using DAPC showing variation between *S. lewini* individuals (dots) and populations (colours) for 9 microsatellite loci.

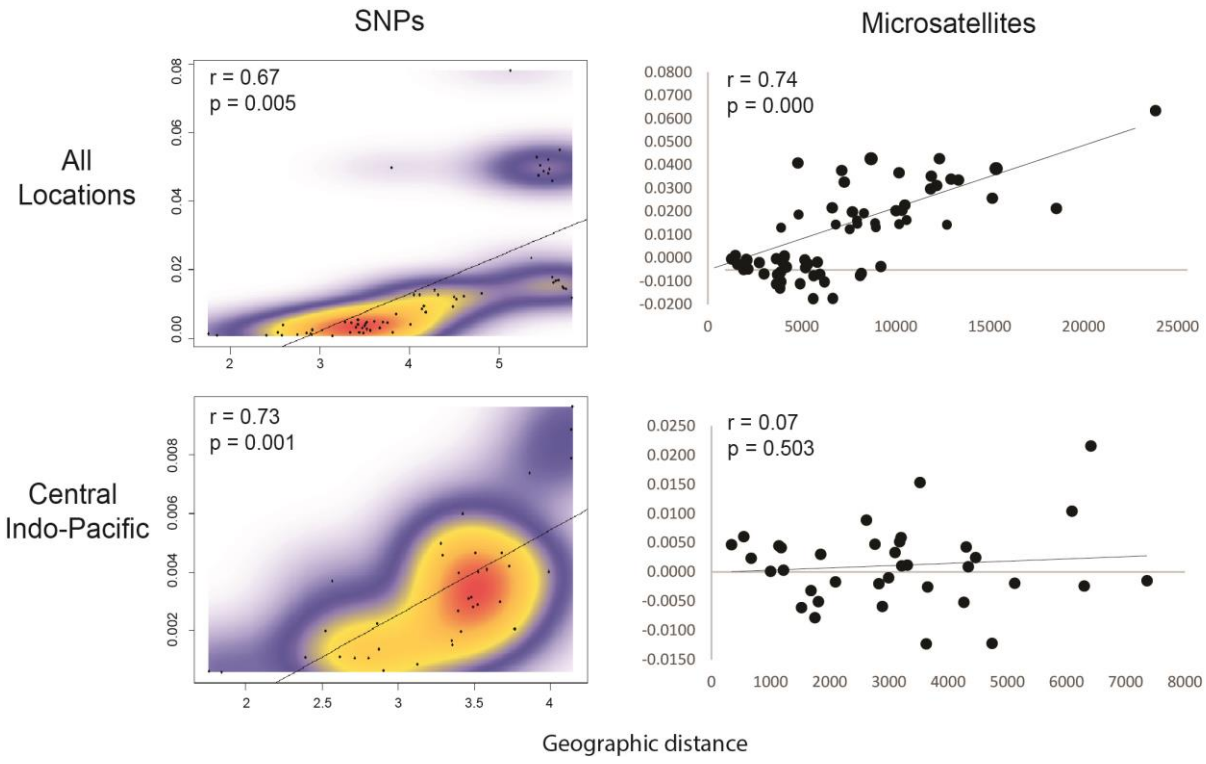

Figure 3.5. Isolation By Distance (IBD) plots showing the relationship between genetic distance (y-axis) and geographic distance (x-axis) for SNPs (left) and microsatellites (right) across two population scenarios; all locations (top) and central Indo-Pacific locations (bottom). SNP IBD plots were generated using dartR package (Gruber et al 2018) where geographic distance is represented as the log of distance in meters.

Single Nucleotide Polymorphisms

DArTSeq processing returned a total of 53,729 SNP loci for 352 individuals from twelve populations. After additional stringent quality filtering (Supplementary Methods) we identified two datasets containing 5,689 and 5,969 SNPs (supplementary Figure B.2, Table B.2). Summary statistics are reported for the total SNP dataset (5,689) in Table 3.1. The number of polymorphic loci per population was similar for most locations, however PNG and GOC had the highest (5,315) and lowest (3,236) respectively (Table 3.1). As expected for bi-allelic SNP markers, A_R and H_O did not vary greatly between locations (A_R = 1.91-1.93, H_O = 0.116-0.130). Similarly to Msats, POWSIM estimates indicated the suit of SNPs used for analyses would be sufficient to detect population differentiation for F_{ST} values between 0.0004-0.05 (power to detect = 1) (Supplementary Table B.5).

Pairwise comparisons were similar between SNPs and Msats with the global F_{ST} being the same (F_{ST} = 0.010). Again, the easternmost location, GOC was the most genetically differentiated from all locations and F_{ST} values were comparable (albeit slightly higher) with Msats (F_{ST} = 0.044-0.072) (Figure 3.3, Table B.6). Comparisons between *S. lewini* from SEY and HAW to all other locations revealed a lack of gene flow with moderate and significant F_{ST} values (F_{ST} = 0.009-0.023, p = < 0.001). Despite somewhat similar trends in F_{ST} values, SNPs and Msats differed greatly in the statistical significance of comparisons. The majority of pairwise tests for SNPs were found to be significant (p = < 0.001), with the exception of closely located regions in Indonesia, Australia and PNG - IN, NT, PCB, NSW, TSV and PNG. The central Indo-Pacific SNP set estimated similar pairwise F_{ST}'s as the all location SNPs with the largest difference between F_{ST} values being +/- 0.001 (supplementary Table B.7).

The unsupervised clustering algorithm run using ADMIXTURE software yielded more visibly structured clusters based on geographic location than STRUCTURE plots for the Msats (using all location SNP set). While the CV error value indicated K = 1 as the most likely genetic clustering scenario, ADMIXTURE plots of K = 2-6 shows distinct clustering based on location (Figure 3.6). Supporting pairwise F_{ST} tests indicating genetic distinctiveness, SEY, GOC and HAW all belong to clearly defined clusters from K = 3 onwards. This is different from Msat STRUCTURE output where HAW and SEY did not form distinct clusters. ADMIXTURE plots also show a high level of homogeneity among many of the central Indo-Pacific locations (PHTW, IN, WA, NT, PNG, PCB, TSV, NSW and FJ) sharing assigned clusters. DAPC plots also support the findings of the pairwise F_{ST}'s and ADMIXTURE analyses for SNPs. Clustering is consistent with geographic locations showing SEY, GOC and HAW most clearly separated from a central Indo-Pacific cluster (Figure 3.7). Testing exclusively central Indo-Pacific locations (5,969 SNPs) we found PHTW, WA and FJ cluster slightly away from other

overlapping locations. Contrary to the Msat results, all population scenarios found significant correlations between genetic and geographic distance. Correlation value (r) varied between groupings with all locations and central Indo-Pacific locations having r values of 0.67 and 0.73 respectively (Figure 3.5).

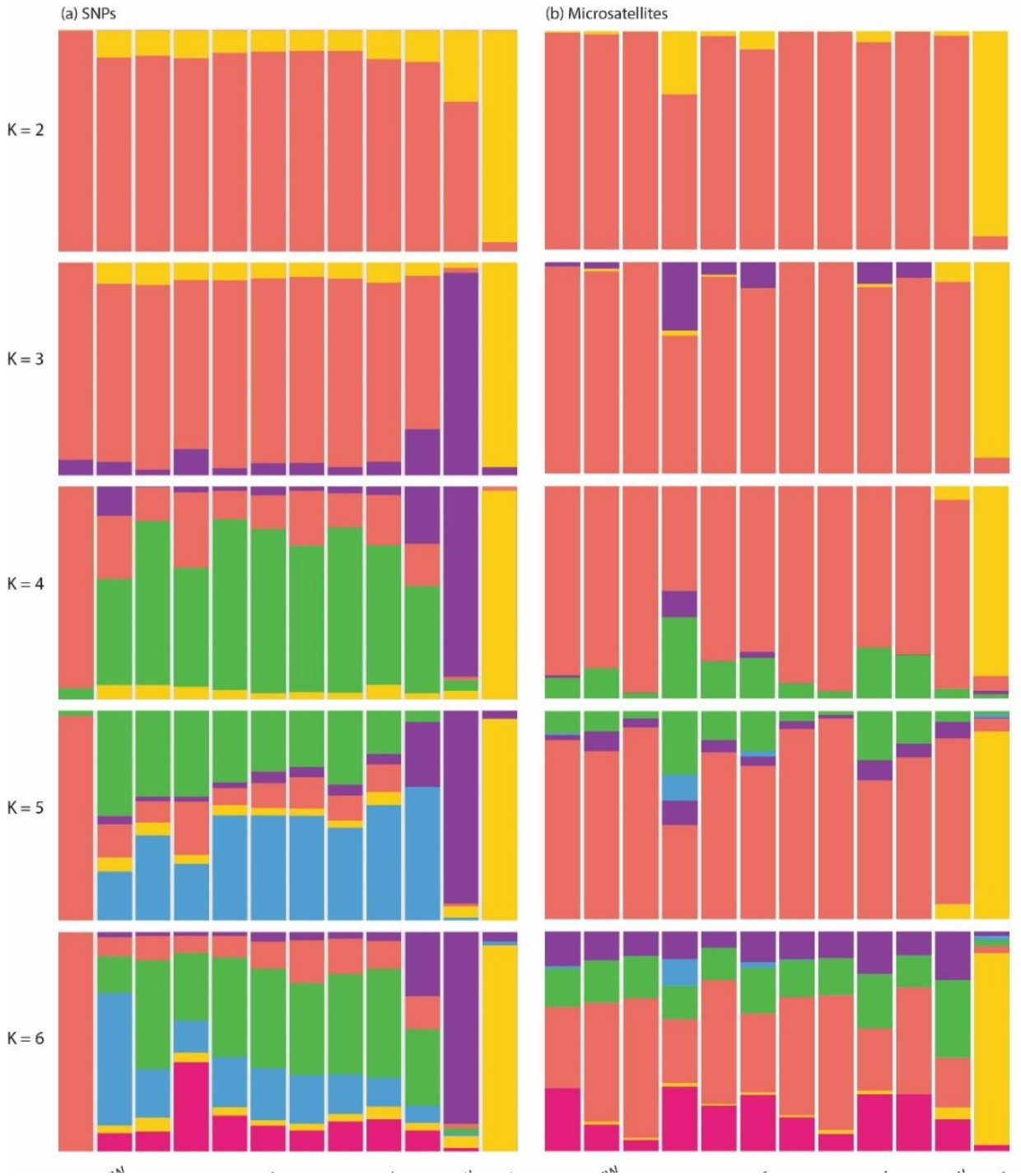

Figure 3.6. Average population for *S. lewini* clustering based on ADMIXTURE (SNPs) and STRUCTURE (Microsatellite) outputs for 5,689 SNPs (left) and 9 microsatellites (right) respectively. Colours represent different clusters as defined by K values. Each column represents a different location.

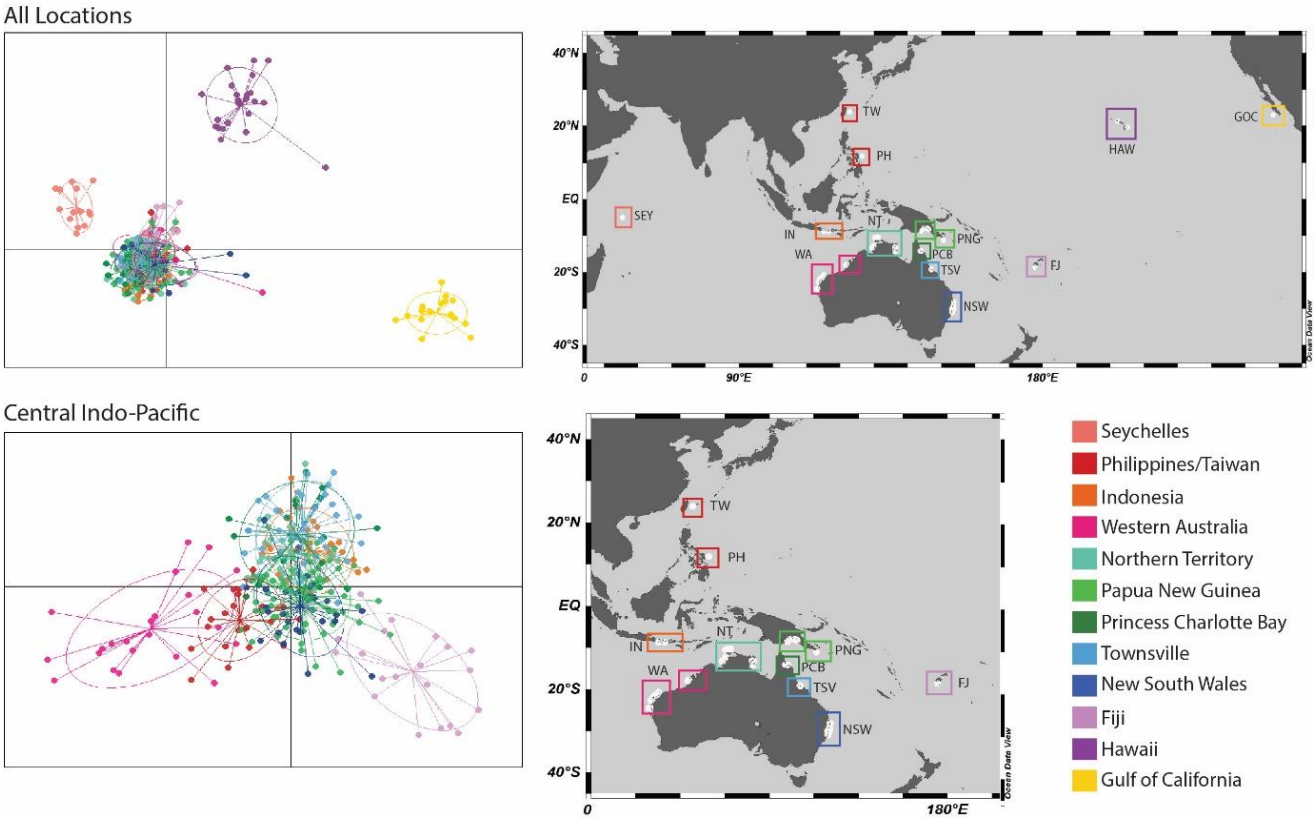

Figure 3.7. Scatterplot created using DAPC showing variation between *S. lewini* individuals (dots) and populations (colours) for two SNP datasets with accompanying map of locations. Top- All locations (5,689 SNPs), bottom- central Indo-Pacific locations (5,969 SNPs).

3.4 DISCUSSION

Population subdivision identified in our study using three marker types has updated our understanding of *S. lewini* dispersal across the Pacific and Indian Oceans. The results presented here clearly show little gene flow is occurring between the westernmost and easternmost populations Seychelles, Hawaii and the Gulf of California with central Indo-Pacific locations (Australia, Indonesia, Philippines, Taiwan, Papua New Guinea and Fiji). The large geographic separation between these outer regions (SEY, HAW & GOC) likely corresponds to the genetic structure identified in our study. The continental shelves of Australia, Papua New Guinea and Indonesia provide well connected

available habitat enabling dispersive behaviours between the Pacific and Indian Oceans and likely drive the high gene flow identified in the central Indo-Pacific region.

This study uses a number of samples and similar genetic markers from previous work including Duncan et al., (2006 b), Daly-Engel et al., (2012) and Ovenden et al., (2009) as well as newly collected samples from Seychelles, Papua New Guinea and Townsville (Australia). Overall, our results are similar, finding structure between ocean basins and connectivity along continental shelves using mtDNA. Additionally, our findings of ocean basin and Indo-Pacific connectivity identified using Msats is similar to descriptions in Daly-Engel et al., (2012) and Ovenden et al., (2009) respectively. The new information in this study, based on the addition of over 5,600 SNP markers have enabled direct comparisons on previous interpretations of *S. lewini* connectivity across all twelve locations. Unlike studies using nuclear DNA in the form of Msats which described genetic homogeneity for *S. lewini* across the Indian and Pacific oceans (likely driven by male biased dispersal) (Daly-Engel et al., 2012) SNPs did detect a lack of oceanic basin connectivity.

Of great interest is the haplotypes thought only to exist in the Atlantic Ocean, identified in individuals from the other locations. Previously the divergent haplotype described for individuals from North/South Carolina, Florida, Louisiana and the Ivory Coast led authors to suggest Atlantic *S. lewini* are a distinct divergent lineage (Quattro et al., 2006). However, Duncan et al., (2006 b) and Daly-Engel et al., (2012) did make comment of a western Indian Ocean haplotype clustering within the Atlantic lineage. In the current study, when focussing on mtDNA CR haplotypes from individuals sampled in Seychelles, mtDNA data demonstrated all individuals from the Seychelles were represented by the same 'Atlantic' haplotype over two separate time periods of sampling (2010 and 2017). Additionally, one individual from Indonesia and PNG and, 3 individuals from the Philippines all had similar haplotypes to the Atlantic lineage. Given the evidence suggesting *S. lewini* centre of origin was out of the Indo-Pacific (Duncan et al., 2006 b), it is likely the Atlantic haplotype could have originated in the Indo-Pacific and through migration west-ward past South Africa moved into the Atlantic. Therefore, the Atlantic haplotype should be reconsidered as more wide spread than initially thought.

Indian and Pacific Ocean population structure

The findings of significant population structure between the Seychelles and Hawaii with the central Indo-Pacific clearly defined using SNPs adds new knowledge to our understanding of *S. lewini* dispersive behaviour. Until now, Msats have only detected population structure between the region

of the Gulf of California and other locations used in this study. Previously, Seychelles and Hawaii were found to be connected with central Indo-Pacific locations, suggesting some level of male-mediated connectivity is occurring across ocean basins (Daly-Engel et al., 2012). Using SNP markers, our results identified individuals from the Seychelles and Hawaii belonging to distinct clusters corroborated with large and significant pairwise F_{ST}'s (analogous with mtDNA results presented here). Similarly, SNP DAPC and ADMIXTURE plots clearly define Seychelles and Hawaii as separate populations from the central Indo-Pacific. Therefore, connectivity of *S. lewini* across the Pacific and Indian Oceans may be more restricted than initially proposed by Daly-Engel et al., (2012).

Overall, K values estimated using ADMIXTURE and STRUCTURE for SNPs and Msats respectively were not concordant. Differences in the number of loci and therefore informativness of each marker likely attribute to varied clusters/populations identified (Morin et al., 2004). Bayesian clustering algorithms are reported to be inaccurate in the presence of low levels of genetic differentiation (Latch et al., 2006), therefore many scenarios of K need to be modelled to assess for biological importance. Scenarios of K = 4-6 populations using SNPs appear reasonable, with the Seychelles, Hawaii and Gulf of California all distinct populations and the central Indo-Pacific (9 locations) making up a homogeneously mixed population. Philippines, Taiwan, Western Australia and Fiji begin to differ at K = 6, however this differentiation is only slight. DAPC and pairwise F_{ST}'s support K scenarios identifying Seychelles, Hawaii, Gulf of California, Fiji and some comparisons between Philippines, Taiwan and Western Australia to be genetically distinct. We therefore propose four major genetic stocks across the Pacific and Indian Oceans; 1. West Indian (SEY), 2. Central Indo-Pacific (PNG, PHTW, IN, WA, NT, PCB, TSV and NSW), 3. Central Pacific (HAW), and 4. East Pacific (GOC). We also note structure occurring within the central Indo-Pacific (i.e. Western Australia and Fiji), however this is less clear among results and the subtle signal of structure is discussed in the section below. The genetic stock groupings suggested here are largely ocean basin focused. A number of other shark species have been found to have limited gene flow across ocean basins (using Msats), including the lemon shark *Negaprion brevirostris* (Schultz et al., 2008), common black tip shark *C. limbatus* (Keeney et al., 2006) and tiger shark *Galeocerdo cuvier* (Bernard et al., 2016). Similar to *S. lewini*, each of these species rely on coastal and/or reef habitat for food, protection and reproduction, the strong reliance on these habitats for critical physiological and ecological functions likely explain why large scale oceanic movements are rare (Ketchum et al., 2014).

It would be beneficial to link genetic findings with movement data, similar to that of Corrigan et al., (2018) who combined genetic and telemetry methods to describe connectivity of shortfin mako sharks *Isurus oxyrinchus* across the Pacific and Indian Oceans. However much of the available telemetry and mark-recapture studies have exclusively captured young-of-the-year and

juveniles from Hawaii (Clarke, 1971; Klimley et al., 1981; Holland et al., 1993; Kohler et al., 2001; Lowe, 2002; Duncan et al., 2006 a) and the South African coast (Diemer et al., 2011). Adult movements have been tracked within the Galapagos Islands finding the majority of individuals to remain resident at tagged reefs (Ketchum et al., 2014). A few large females moved significant distances (~700kms) and returned to resident reefs. Small movements between closely located islands was suggested to be foraging behaviour off sea mounts and was correlated with strong currents (Ketchum et al., 2014). Demographic movements of adult *S. lewini* in more regions need to be assessed, however locating adults and their propensity for post-release mortality make tagging efforts challenging.

Genetic diversity and structure in the central Indo-Pacific

Locations outside of the central Indo-Pacific were removed (Seychelles, Hawaii and Gulf of California) and SNPs were re-filtered and analysed to estimate fine scale structure within the connected region of the central Indo-Pacific. Secondary filtering for central Indo-Pacific locations yielded a total of 5,969 SNPs with 931 of these exclusive to the fine scale dataset. Regional assessments of *S. lewini* in the central Indo-Pacific is important given the data deficient status in Oceania and closely located EEZ's of Indonesia, Papua New Guinea and Australia.

SNPs, Msats and mtDNA identified some level of shared gene flow across the central Indo-Pacific consisting of a number of countries; Australia, Papua New Guinea, Indonesia, Philippines, Taiwan and Fiji. Connectivity across the Indo-Pacific has been noted for *S. lewini* previously (Daly-Engel et al., 2012; Ovenden et al., 2009) as well as a number of other shark species including *G. cuvier* (Holmes et al., 2017), short fin mako *Isurus oxyrinchus* (Corrigan et al., 2018), *C. amblyrhynchos* (Momigliano et al., 2017), *C. albimarginatus* (Green et al., 2019) and *P. glauca* (Ovenden et al., 2009). Within the central Indo-Pacific region, *S. lewini* are continuously distributed (Last et al., 2009) with no perceived contemporary barriers hindering dispersal along continental shelves.

Interestingly, SNP markers reveal a subtle level of population structure within the central Indo-Pacific. The majority of SNP pairwise comparisons between central Indo-Pacific locations are significantly different, suggesting gene flow between these locations may be limited. Only some of the geographically closest locations including the Northern Territory, East Australian coast, Indonesia and PNG comparisons are not significantly partitioned, likely driving the observed pattern of Isolation By Distance (IBD). Supported by F_{ST}, DAPC and ADMIXTURE plots, our results suggest Fiji,

Western Australia and Philippines/Taiwan have restricted gene flow between other central Indo-Pacific locations. Given the presence of IBD driving genetic diversity patterns among the central Indo-Pacific, it is likely gene flow across the region is facilitated by stepping stone migrations as was the case for *C. amblyrhynchos* (Momigliano et al., 2017). Reef coverage is continuous between eastern/northern Australia, PNG and Indonesia, however coral reef habitat in Western Australia is sparser, separated by hundreds of kilometres of unsuitable habitat (Momigliano et al., 2017). The distance and lack of suitable reef structure between Western Australia, Fiji and Philippines/Taiwan and other central Indo-Pacific locations may reduce dispersal and therefore gene flow between these locations. In contrast, high gene flow identified between east/north Australia, PNG and Indonesia suggests movement of *S. lewini* between these locations is likely.

Microsatellite vs SNP markers for population assignment

Results between nuclear markers Msats and SNPs largely agree, however a few differences were identified. First, the clusters inferred by SNP DAPC are much more distinct then Msats. Second, while global F_{ST} values were the same and pairwise F_{ST}'s followed similar trends, their significance was highly varied. Third, the IBD pattern in the central Indo-Pacific was much stronger in the SNP data. Forth, assignment of clusters using Bayesian programs ADMIXTURE and STRUCTURE identified different K values and population assignment between K = 1-6 varied, with SNPs more accurately assigning individuals to their geographic locations. A number of studies comparing SNPs and Msats have found similar patterns with SNPs more clearly defining DAPC and PCA clusters (Benestan et al., 2015; Malenfant et al., 2015; Jeffries et al., 2016), IBD correlations (Coates et al., 2009; Jeffries et al., 2016) and larger, significant F_{ST} values (Malenfant et al., 2015; Vendrami et al., 2017). These differences can be attributed to SNPs more densely sampling the genome, identifying a large subset of loci, highly differentiated and providing fine-scale resolution and estimations of population structure (Xing et al., 2005; Hohenlohe et al., 2018). Estimates from POWSIM suggested Msats used in our study were sufficient to recognise population differentiation between F_{ST} = 0.001-0.05, however this was not the case and the majority of pairwise F_{ST} values were found to be non-significant. This discrepancy is likely from differences between simulated and empirical datasets, including possible non-independent effective gene flow occurring between populations. The significance of SNP F_{ST} values is likely due to the sheer number of SNP loci lowering residuals and creating more confidence in calculated observations. Similar to many other studies before us, we have shown SNPs to provide fine-scale discrimination of population structure for a widely distributed marine species where weakly differentiated Msats were not capable.

Management Implications and Conclusion

Genetic analysis of population subdivision provides important information for fisheries management. If population structure is identified this can be used to aid defining a geographic boundary of a biological stock (Ovenden et al., 2009). Using results from this study we have defined the observed biological stock structure into four regions; the West Indian (Seychelles), the central Indo-Pacific (Papua New Guinea, Indonesia, Australia, Philippines, Taiwan and Fiji), Central Pacific (Hawaii) and the Tropical East Pacific (Gulf of California). As was completed for the central Indo-Pacific, it would be of great interest to estimate intra-regional connectivity between closely positioned locations in the West Indian (around the Seychelles) and east Pacific (around Gulf of California). Future studies should incorporate samples near regions tested within our study to determine the extent of connectivity within ocean basins.

Within the central Indo-Pacific, only subtle population structure has been identified; connectivity appears to be present between east and north Australia, PNG and Indonesia, however small breaks in gene flow are observed between the west coast of Australia and Fiji. Conceptual models previously developed for *S. lewini* to explain patterns of distribution described four possible models of movement (Chin et al., 2017). Using the results of this study we can assume Model 1 and Model 2 suggesting panmixia and limited movement respectively are unlikely patterns of connectivity. Instead, data reported here would support Model 3; Continental shelf movement enabling connectivity between Australia, PNG and eastern Indonesia, but not pacific islands (i.e. Fiji). In addition to Model 3, the results suggest connectivity to Western Australia appears to be limited. Adopting management at the spatial scales and boundaries identified in this study in particular for the central Indo-Pacific region will include international and national cooperation.

A key objective of this study was to assess whether differences in biological stock structure could be observed using a multi-marker approach. The work presented here indicates differences between Msats and SNPs do occur, with SNPs identifying more discrete population subdivision than Msats. The ability of genomic techniques to capture a large subset of highly differentiated markers provides a robust approach to identify population structure (Hohenlohe et al., 2018). These results suggest increased sampling regimes or loci are required if choosing to undertake population structure analyses exclusively with Msat markers. Therefore, undertaking a genomic approach using SNPs may be more suited for shark and ray population structure studies given the challenges faced (expense and accessibility) when obtaining adequate sample sizes. Future studies assessing population connectivity using alternative demographic methods (i.e. telemetry, parasites) should be

undertaken in order to estimate the level of demographic connectivity between nations with varying levels of fishing pressure and capacities for management

Chapter 4 Variability in multiple paternity rates for grey reef sharks (*Carcharhinus amblyrhynchos*) and scalloped hammerheads (*Sphyrna lewini*)

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4.1 INTRODUCTION

Increasing resolution of molecular tools allows for a greater understanding of shark and ray (elasmobranch) reproductive systems which are often difficult to observe in the wild (Feldheim et al., 2001; Chapman et al., 2004; Whitney et al., 2004). Elasmobranchs exhibit a variety of reproductive modes including live-bearing (viviparity), egg laying (oviparity) (Wourms, 1977) and parthenogenesis (Dudgeon et al., 2017) and also display monogamous and polyandrous mating behaviours (Feldheim et al., 2007; Portnoy et al., 2015). Elasmobranchs do not often form pairs before and/or after mating and do not provide postnatal care to offspring (Pratt et al., 2001), making their propensity for behavioural monogamy generally low. Instead, it is more likely for females to display polyandrous behaviour, mating with a number of males (Carrier et al., 1994; Pratt et al., 2001), the outcome of which may be a single litter, sired by many males and composed of full and half-siblings (sibs) (i.e. multiple paternity) (Birkhead et al., 1998). Polyandry with multiple paternity has a number of benefits (Sugg et al., 1994; Chapman et al., 2004; Neff et al., 2005; Daly-Engel et al., 2006). Firstly the fitness of the mother is increased as she is more likely to produce offspring; secondly, the adaptive fitness of individuals within litter may be improved as genetic variation is more likely to increase; thirdly, increases in genetic diversity can counteract issues of inbreeding facilitated by close-kin mating (especially for small populations); and finally, multiple paternity can increase the effective population size by providing an opportunity for a greater number of males to mate with an increased number of females (Chapman et al., 2004).

The occurrence and prevalence of multiple paternity within an elasmobranch litter varies between species, populations and even individuals, but reasons for this are poorly understood (Chapman et al., 2004; Byrne et al., 2012). Previous studies have suggested the likelihood of genetic monogamy or polyandry within a litter is dependent on a number of factors including the mother's size, home range or philopatric tendencies, population size, species-specific behaviours and the presence of post copulatory mechanisms (e.g. sperm storage) (Chapman et al., 2004; Feldheim et al., 2004; Neff et al., 2005; Portnoy et al., 2007; Daly-Engel et al., 2010; Byrne et al., 2012; Boomer et al., 2013).

Sharks have life-history characteristics that make them highly susceptible to population declines, e.g. slow growth, delayed maturation and low fecundity (Last et al., 2009; Dulvy et al., 2014). An estimated 25% of all shark and ray species are threatened under the criteria of the International Union for Conservation of Nature (IUCN) Red List, with overfishing considered one of the main causes (Dulvy et al., 2014). Population assessments require knowledge of shark demographics including mating systems, thus determining the extent of multiple paternity is useful for developing long term management and conservation plans for sharks (Pratt et al., 2001; Rowe et al., 2003; Byrne et al., 2012).

In Papua New Guinea (PNG), grey reef sharks (*Carcharhinus amblyrhynchos*) and scalloped hammerheads (*Sphyrna lewini*) are commonly caught by coastal artisanal and commercial fisheries. Regionally, the level of exploitation of both species is undocumented, making it difficult to assess the status of local populations. Globally, overexploitation has led to international conservation measures for *S. lewini* (i.e. listed as Endangered on the IUCN Red List (Baum et al., 2007) and included in Appendix II of the Conservation on International Trade in Endangered Species), while *C. amblyrhynchos* are recognised as Near Threatened (IUCN Red List), thereby demonstrating the capacity to recover if managed accordingly (Smith et al., 1998).

Each species, *C. amblyrhynchos* and *S. lewini* differ ecologically; while both have overlapping distributions, their habitat usage differs. *C. amblyrhynchos* have a strong affiliation with reef systems and often smaller individuals will show signs of site attachment to specific reefs (Heupel et al., 2010). Furthermore, telemetry studies have identified sex-specific movement traits for *C. amblyrhynchos*, with males more likely to travel to neighbouring reefs than females (Espinoza et al., 2014 b). *S. lewini* display more complex habitat usage patterns including large ontogenetic differences and broader sex-specific movement traits (Klimley, 1985, 1987). Generally, juvenile *S. lewini* are found in shallower inshore waters, while adults migrate to deeper continental shelf environments (Klimley 1987). Genetic analyses suggests females are more likely to display philopatric tendencies, adhering to coastal habitats, while males are known to disperse across oceans (Daly-Engel et al., 2012). Both *C. amblyrhynchos* and *S. lewini* form large female aggregations (Klimley, 1987; Economakis et al., 1998) and, once gravid, they are known to move inshore seeking refuge in nursery areas for birthing (Daly-Engel et al., 2012). Additionally, *S. lewini* have post copulatory mechanisms allowing for long-term (months to years) (Demski et al., 1993) sperm storage.

Obtaining mother and litter information for sharks is challenging; only a limited number of multiple paternity studies have been undertaken on elasmobranchs and often sample sizes are limited (Chapman et al., 2004; Rossouw et al., 2016). Recently, MP analyses were undertaken for *S.*

lewini in southern Africa (Rossouw et al., 2016). Using up to six microsatellite loci, Rossouw et al., (2016) identified MP in 46% of 13 litters tested. Conversely, there has been no assessment of multiple paternity in *C. amblyrhynchos* from any location. Here we investigated MP in *C. amblyrhynchos* and *S. lewini* captured in the Indo-Pacific Ocean. Given that all studies which have undertaken paternity tests on shark litters have uncovered MP (see review in Rossouw et al., 2016) we predict multiple paternity will also be found for both species in this current study. However rates are likely to differ given the variation in behaviour, ecology and physiology between the species. Using suites of microsatellite markers, litters were genetically determined as consisting of full or half sibs with an estimate of the number of fathers and their contribution to the litters in each species also obtained. This is the first study to investigate multiple paternity in *C. amblyrhynchos* and the first for *S. lewini* in the Indo-Pacific Ocean.

4.2 METHODS

Sampling and Microsatellite Analyses

Sample collection was undertaken on board commercial fishing vessels operating in PNG between 3rd May 2014 and 6th June 2014. Sampling was undertaken by observers deployed as part of an Australian Centre for International Agricultural Research project led by the National Fisheries Authority (NFA) of PNG and CSIRO to assess shark and ray catches throughout the commercial and artisanal fisheries in PNG (experiments approved by ACIAR and CSIRO; project FIS/2012/102). All samples were collected within a single month from the Bismarck and Solomon Seas (Figure 4.1). Tissue samples including fin clips, vertebral chord or muscle were collected from pregnant females and all pups. Observers recorded total length of the adult females and measurements from the smallest and largest pups within a litter.

DNA was extracted using the Wizard© SV Genomic DNA Purification system (Promega, Australia); tissue extractions were undertaken using SV minicolumns following modifications to the manufacturer's instructions (i.e. overnight tissue digestion; amount of supernatant used to elute DNA was reduced; DNA elution times increased). DNA was quantified using a Nanodrop 8000 UV-Vis Spectrophotometer (Thermo Scientific, USA) and standardised to 20ng/uL.

Microsatellites from pups in each litter were amplified by Polymerase Chain Reaction (PCR) and compared to genotypes in the corresponding mother. Species-specific microsatellite primers for *C. amblyrhynchos* and *S. lewini* were from Momigliano et al., (2014) and Nance et al., (2009) respectively. In the current study, microsatellite multiplexes were developed to enable cost effective

screening. Forward primers were labelled with 6-FAM, VIC, NED and PET proprietary dyes and multiplexed (Table 4.1). PCR reactions consisted of GoTaq© Colourless Master Mix (Promega, USA), Bovine Serum Albumin (Promega, USA), 0.2µM of each individual F and R primer (see Table 4.1 for multiplexes), and 0.8ng/µl DNA in a 25µL reaction. For *S. lewini*, thermal cycling consisted of initial denaturation at 94°C x 3 minutes, 35 cycles of 94°C x 1 minute, 58°C x 30 seconds, 72°C x 1 minute and a final extension of 72°C x 10 minutes. Thermal cycling for *C. amblyrhynchos* consisted of a touch-down protocol including initial denaturation at 94°C x 3 minutes, 35 cycles of 94°C x 1 minute, 5 cycles of 56°C x 30 seconds, 5 cycles of 54°C x 30 seconds, 25 cycles of 52°C x 30 seconds, 35 cycles of 72°C x 1 minute and a final extension of 72°C x 10 minutes. Following PCR amplification, Gene Scan™ LIZ 500® size standard (Thermofisher Scientific, USA) and formamide were added to 3 µL of each PCR reaction and 20µL sample volumes were run on an ABI 3130XL AutoDNA sequencer (Thermofisher, USA). Genotypes were scored and checked by eye using Geneious© R8.1.4 Microsatellite plug-in program (Biomatters Ltd Auckland, New Zealand).

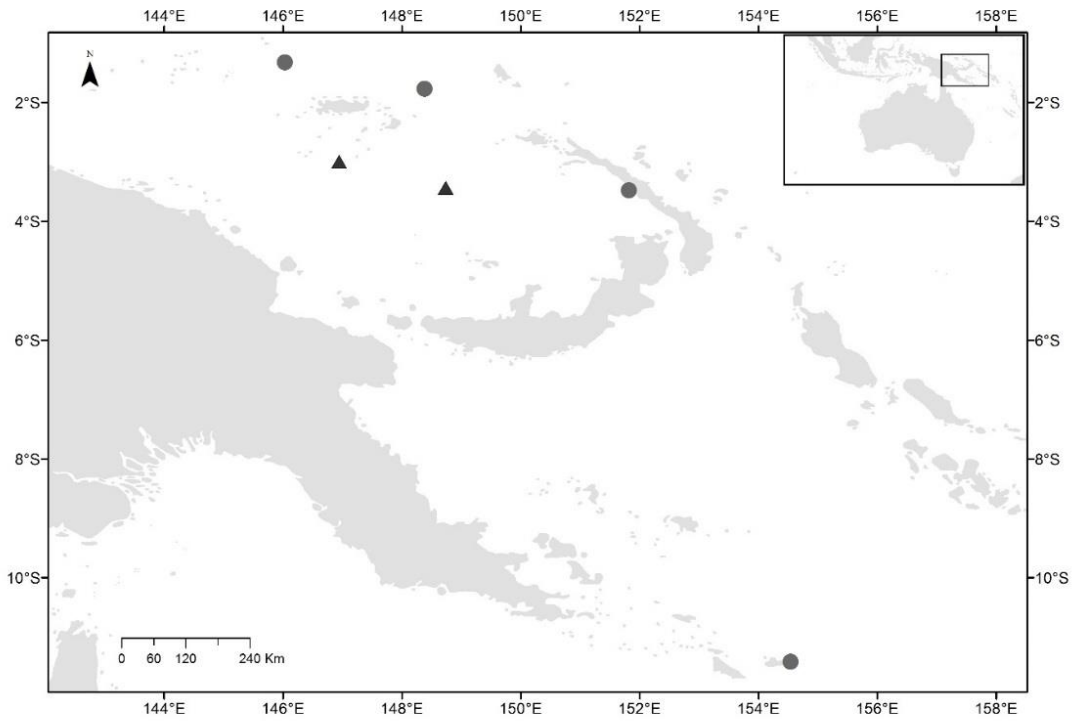


Figure 4.1. Sample locations for C. amblyrhynchos (circles) and S. lewini (triangles) in Papua New Guinea. Map created using ArcMap 10.2.1 (http://desktop.arcgis.com/en/arcmap/).

Table 4.1. Characterisation of microsatellite loci for *C. amblyrhynchos* and *S. lewini*.

Locus Name	n	N_a	H_o	H_e	PIC
C. amblyrhynchos	26				
C.amb11[1]		14	0.938	0.895	0.878
C.amb3[1]		26	0.844	0.921	0.908
C.amb7[1]		8	0.703	0.759	0.715
C.amb2[1]		13	0.887	0.883	0.863
C.amb27[2]		10	0.797	0.823	0.793
C.amb9[2]		6	0.641	0.601	0.530
C.amb28[2]		12	0.844	0.807	0.779
C.amb4[2]		16	0.828	0.81	0.782
C.amb18[3]		25	0.938	0.952	0.942
C.amb15[3]		15	0.746	0.865	0.842
C.amb5[3]		9	0.813	0.766	0.726
C.amb22[3]		4	0.094	0.134	0.129
C.amb25[4]		10	0.906	0.826	0.797
C.amb20[4]		14	0.828	0.883	0.863
S. lewini	91				
SLE027[1]		9	0.867	0.804	0.773
SLE018[1]		4	0.545	0.516	0.472
SLE089[1]		18	0.966	0.91	0.898
SLE038[2]		7	0.943	0.781	0.744
SLE045[2]		4	0.818	0.721	0.665
SLE054[2]		5	0.685	0.664	0.621
SLE053[3]		12	0.667	0.84	0.817
SLE081[3]		8	0.922	0.787	0.753
SLE071[3]		11	0.582	0.738	0.713
SLE077[3]		13	0.681	0.889	0.873

Number of individual mothers and pups (n), number of alleles (N_A), observed heterozygosity (H_o), expected heterozygosity (H_e) and Polymorphic Information Criteria (PIC).

Statistical Analysis

For each microsatellite locus, numbers of alleles, allele frequencies, and observed (H_o) and expected heterozygosities (H_e) were determined using Genepop web service v4.0.10 (Rousset, 2008). Significance of H_o and H_e tests were estimated by the Markov Chain method including 10,000

dememorizations, 500 batches and 10,000 iterations (not reported). Polymorphic information content (PIC) was estimated using Cervus v3.0 (Kalinowski et al., 2007).

Analysis of paternity was initially checked by visual inspection of multi-locus genotypes. Secondly, putative fathers (number of sires) and paternal skew within litters were inferred using two programs: Gerud v2.0 (Jones, 2005) which identifies the minimum number of fathers through exclusion calculations, and Colony v2.0.4.5 (Jones et al., 2010) which uses a maximum likelihood approach. Polygamous mating systems were assumed for both sexes to allow for the assignment of full and half-sibs in Colony. Probability of detecting multiple paternity was calculated post-hoc using PrDM software (Neff et al., 2002) (available at http://publish.uwo.ca/~bneff/software.html). Six different scenarios were tested and defined according to the number of pups per litter and the minimum number of fathers identified in Gerud v2.0 (Jones, 2005). These scenarios were defined according to the number of pups observed in the present study (for each species) and the degree of paternity tested in other shark PrDM MP analyses (Chapman et al., 2004; Feldheim et al., 2004; Daly-Engel et al., 2006).

4.3 RESULTS

Six litters of *C. amblyrhynchos* and five litters of *S. lewini* were used to investigate the presence of multiple paternity for sharks captured in PNG waters. Litter size between the species was significantly different (P = 0.007, Wilcoxon rank sum test), with *C. amblyrhynchos* having an average litter of 3.3 pups and *S. lewini* an average of 17.2 (Table 4.2). Sex ratios within litters showed no significant bias towards either sex ($p > 0.05$, chi-square test). Litter size was positively correlated with adult female length for *S. lewini* (p = 0.023, R^2 = 0.859, Pearson's rank correlation) but not for grey reefs (p = 0.675, R^2 = 0.000) (Figure 4.2). We note however, that these correlation analyses are based on small sample sizes (i.e. litter numbers per species) and should be treated with caution.

Alleles were amplified in microsatellite suites of 14 and 10 loci for all mothers (genotyped twice) and pups across 26 *C. amblyrhynchos* and 91 *S. lewini*, respectively (Table 4.1). As Table 4.1 shows, H_o ranged from 0.094 – 0.938 in *C. amblyrhynchos* and 0.545 – 0.966 in *S. lewini*. PIC values were generally high, with 86% and 70% of *C. amblyrhynchos* and scalloped hammerhead loci greater than 0.7 respectively. The probability of detecting multiple paternity (PrDM) was highest for *S. lewini* (0.94 to 1), while probabilities were varied and reduced for *C. amblyrhynchos* (0.47 to 1; Table 4.3). Number of loci had less effect than the number of pups within a litter in the detection of multiple paternity. Multiple paternity was identified in 66% of *C. amblyrhynchos* litters (4 out of 6) and in all scalloped hammerhead litters (all five) (Table 4.2). The number of putative fathers ranged from 1–3

for *C. amblyrhynchos* and 2–8 for *S. lewini* based on Gerud and Colony estimates. In most cases, Colony analysis detected the same or a higher number of sires than Gerud. Paternal skew was identified in two scalloped hammerhead litters indicating an uneven contribution of pups per sire (Table 4.2).

Table 4.2. Summary of analysed litters, including female total length, litter size, sex ratio of pups (M:F Ratio), size range of pups, number of sires as estimated by Gerud and Colony, skew (paternal) *C. amblyrhynchos* and *S. lewini*.

Species	Total Length (cm)	Litter Size	M:F Ratio	Size range of pups (cm)	# Sires (Gerud)	Skew (Gerud)	# Sires (Colony)
C. amblyrhynchos	160	4	3:1	51-54	2	2:2	2
C. amblyrhynchos	160	5	3:2	52-56	2	3:2	3
C. amblyrhynchos	153	3	0:3	40-41	2	2:1	2
C. amblyrhynchos	158	3	1:2	54-56	1	-	1
C. amblyrhynchos	150	2	1:1	45-62	1	-	1
C. amblyrhynchos	177	3	3:0	20-21	2	2:1	2
S. lewini	249	18	8:10	46-50	3	6:10:2	8
S. lewini	292	25	17:8	44-51	3	5:17:3*	7
S. lewini	238	13	NA	5-7	4	3:5:3:2	4
S. lewini	209	13	4:9	38-41	2	10:3*	2
S. lewini	235	17	9:8	42-48	4	8:3:4:2	3

NA Indicates pups were too young to identify sex, * $p < 0.05$ chi-square test

Table 4.3. Probability to detect multiple males (PrDM) using different suites of microsatellite markers: 14 loci for *C. amblyrhynchos* and 10 loci for *S. lewini* under a number of paternal skew scenarios.

	Litter Size						
	C. amblyrhynchos			*S. lewini*			
	3	4	5	13	17	18	25
Paternal skew							
2 males (50:50)	0.74	0.88	0.94	1.00	1.00	1.00	1.00
2 males (66.7:33.3)	0.71	0.84	0.91	1.00	1.00	1.00	1.00
2 males (80:20)	0.47	0.59	0.68	0.94	0.98	0.98	1.00
3 males (33.3:33.3:33.4)	0.88	0.96	0.99	1.00	1.00	1.00	1.00
3 males (57:28.5:14.5)	0.78	0.89	0.94	1.00	1.00	1.00	1.00
4 males (25:25:25:25)	0.93	0.99	1.00	1.00	1.00	1.00	1.00

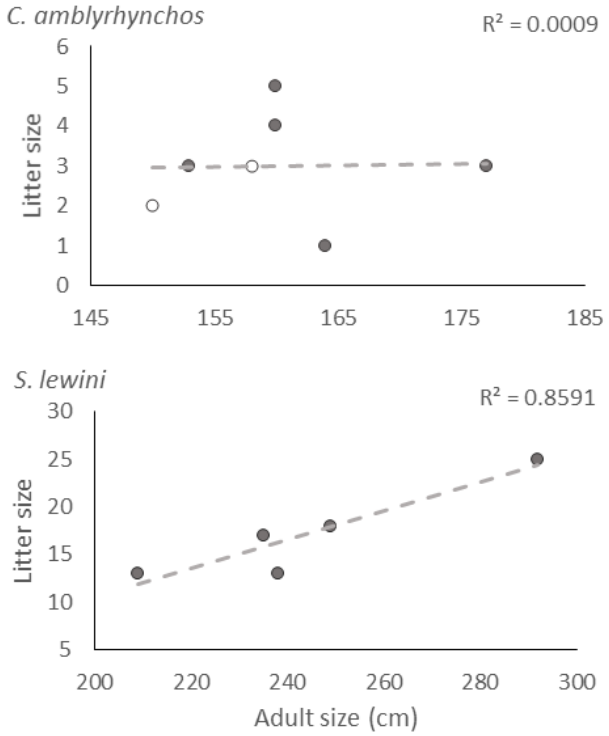


Figure 4.2. Correlation between adult female length (TL) and litter size for grey reef sharks *(C. amblyrhynchos*) and scalloped hammerhead (*S. lewini*). Shaded points indicate litter with multiple paternity, unshaded represents litters without multiple paternity.

4.4 DISCUSSION

Results from this study provide the first evidence of multiple paternity in *C. amblyrhynchos*, and the presence of MP in all studied litters of *S. lewini* in the Indo-Pacific Ocean. This is the first identification of 100% MP for a species of shark (albeit with a limited number of litters, *n* = 5) and the second within all elasmobranchs studied; 100% multiple paternity has previously been identified in the thornback ray *Raja clavata* (Chevolot et al., 2007). Multiple paternity was observed in 66% of *C. amblyrhynchos* litters, but the power to detect multiple paternity decreases with decreasing litter size, as shown in PrDM analyses (Table 3). Given the small litter sizes, it is possible analyses presented here underestimate levels of MP for *C. amblyrhynchos*. Alternatively, we believe small litter sizes may simply create a limited number of embryos available for fertilization by multiple males.

The percentage of litters reported to have MP for *C. amblyrhynchos* (66%) is comparable to that of other large live bearing sharks, including the sandbar shark *Carcharhinus plumbeus* (40%) (Portnoy et al., 2007). The benefits of polyandrous behaviour have been previously described and include ensuring successful fertility, increasing genetic diversity and genetic fitness (of mother and pups), and reducing close-kin mating (important, if populations are small or inbred) (Feldheim et al., 2002; Saville et al., 2002). Our observation that polyandrous mating was detected in the larger of the *C. amblyrhynchos* females may have implications for the *C. amblyrhynchos* population (exploited in PNG waters) (e.g. size specific breeding females impacted by fishing), however as this study was based on small sample sizes, we cannot elaborate further.

The finding of 100% multiple paternity in scalloped hammerhead litters in this study contrasts with another study which identified only 46% multiple paternity across 13 litters in southern Africa (Rossouw et al., 2016). Interestingly, however, Rossouw et al., (2016) reported an average litter size of seven pups, less than half of the average litter size in the current Indo-Pacific study and well below the documented litter size for *S. lewini* in South Africa (*n* = 30) (Bass et al., 1976). Sharks in the Rossouw et al., (2016) study were captured in bather protection nets, and it is possible the mothers may have aborted the majority of pups prior to landing, potentially limiting the study to a subset of all pups in the litter. This could lead to an underestimate of the level of multiple paternity for *S. lewini* in South Africa.

Multiple paternity is thought to be more common in species that display high levels of philopatry and low dispersal rates, as such behaviour is likely to reduce the chance of individuals breeding with a genetically incompatible (related) partner, thereby decreasing the chance of localized inbreeding depression (Sugg et al., 1994; Feldheim et al., 2002; Chapman et al., 2004). For both scalloped hammerhead and *C. amblyrhynchos*, genetic (Daly-Engel et al., 2012; Momigliano et al., 2015) and telemetry studies (Ketchum et al., 2014) have revealed patterns of female mediated site fidelity and male-biased dispersal. Male dispersal has been prevalent enough to facilitate connectivity (gene flow) between reefs spanning 1,200 km for *C. amblyrhynchos* (Momigliano et al., 2015) and across ocean basins for *S. lewini* (Daly-Engel et al., 2012). For both species in PNG, it would seem the presence of MP is unlikely to be driven by the threat of close-kin mating or inbreeding depression, given the significant gene flow facilitated by male dispersal in these species shown elsewhere.

Two of the five scalloped hammerhead litters were identified as having significant paternal skews. The presence of paternal skew, (i.e. the uneven contribution of sires to a litter) is thought to be attributed to a combination of female choice, the timing/order of males mating, and sperm

competition (Boomer et al., 2013; Rossouw et al., 2016). The processes of post-copulatory mechanisms are thought to increase the level of paternal skew within a litter (Jennions et al., 2000; Fitzpatrick, 2012; Marino et al., 2015; Pirog et al., 2015). *S. lewini* have complex oviducal glands capable of stimulating bundles of sperm to be released, giving control over sperm utilization and its contribution to paternal skew within a litter (Demski et al., 1993; Hamlett et al., 2002; Farrell et al., 2014). Additionally, it is thought that polyandrous mating may create an internal environment within a female that promotes sperm competition, leading to increased fertilization and consequently increased fitness of young ('sexy-sperm hypothesis') (Harvey et al., 1989; Egan et al., 2016). This hypothesis suggests females mate with different males to create conditions selecting for the most competitive sperm; which results in male offspring possessing the gene for heightened sperm competitiveness and therefore increasing offspring fitness (Egan et al., 2016). It is possible males with heightened sperm competitiveness would sire more pups within a litter creating paternal skew. The mechanisms behind paternal skew in *S. lewini* could be one or a combination of factors described here and remains unresolved. The observed lack of paternal skew in *C. amblyrhynchos* may be connected to the smaller litter size of the species; more litters are required to conclusively verify this hypothesis.

The results of this research concur with similar studies and reiterate the prevalence of MP in sharks. Our results highlight the difference in litter size between the *C. amblyrhynchos* and *S. lewini* and demonstrates differences in levels of multiple paternity. Additionally, the discovery of positive correlations between adult size, litter size and MP suggests genetic mating systems in sharks are complex and may be species- and location-specific. Sample sizes presented here are relatively small and further investigation is required to conclusively understand the relationship between adult size and breeding behaviours. However, a number of studies assessing multiple paternity in sharks (and elasmobranchs more widely) have tested five or less litters (Feldheim et al., 2001; Saville et al., 2002; Daly-Engel et al., 2006; Chevolot et al., 2007; Hernández et al., 2014) and given the opportunistic nature and difficulties associated with sampling gravid elasmobranchs, the findings from this research provide valuable insight for these two species. Observations from this work in combination with information of size and sex-specific landings for *C. amblyrhynchos* and *S. lewini* can help to identify if, and where, populations of these species are at risk of reduced genetic diversity and potential population declines.

Chapter 5 Final Discussion

5.1 An Overview

In this thesis, through chapters 2 to 4, the analyses have demonstrated that genetic and genomic markers provide powerful tools for delineating biological stock structures and providing insights into mating behaviours of sharks. The thesis has demonstrated similar patterns of gene flow, whereby connectivity is constrained between ocean basins for two large bodied sharks determined using three genetic markers. Novel mtDNA gene sequencing, microsatellite and NGS libraries have been developed and deployed for *C. albimarginatus*, *S. lewini* and *C. amblyrhynchos* (NGS data not shown) providing a robust foundation for future population genetic studies for these species in unstudied areas. The intra-population level analyses of *C. albimarginatus* and *S. lewini* sharks captured in PNG found no genetic population structure apparent within the country's EEZ. Furthermore, the genetic stocks of *C. albimarginatus* and *S. lewini* are estimated to be shared between neighbouring nations; Australia (and Indonesia for *S. lewini*). This thesis also described the presence of multiple paternity in two species captured in PNG, *S. lewini* and *C. amblyrhynchos*, providing contrasting insights into their mating behaviour. Finally, chapters 2 and 3 have provided a robust comparison between commonly used mtDNA genes, microsatellites and recently developed SNP markers. Perhaps most beneficial to the field of elasmobranch population genetics, is the comparison between markers on the same set of samples providing insight for population geneticists to better understand the similarities and differences when using each marker type for biological stock assessment.

5.2 Comparison of genetic and genomic methods

This thesis tested the effectiveness of genetic markers used in two connectivity assessments for *C. albimarginatus* and *S. lewini* and multiple paternity assessments for *C. amblyrhynchos* and *S. lewini*. Key comparisons were made between nuclear markers; Msats and SNPs. Three main types of classical statistical methods were undertaken; PCA based partitioning of a distance matrix (DAPC), model-based clustering (ADMIXTURE, STRUCTURE) and F-statistics including pairwise F_{ST} analyses. Wide-ranging marine taxa are notorious for having low genome-wide F_{ST}s, presenting a challenge to measure breaks in gene flow (Cowen et al., 2006; Allendorf et al., 2010). SNPs are increasingly proving useful to delineate structure and interpreting biological meaning (Nielsen et al., 2009; Lamichhaney et al., 2012). One of the major objectives of this thesis was to assess how using thousands of SNPs could help to better delineate fine-scale genetic structure and increase the

assignment success to predict putative populations in weakly genetically structured shark species. The results suggest the performance of markers to measure connectivity may also depend on the sampling design of a study. There are a number of considerations when selecting a genetic marker for diversity, population structure, relatedness or kinship assessments. These include but are not limited to project objectives, sampling design, required analytical power, project cost and time (Banks et al., 2003; Benestan et al., 2015; Hohenlohe et al., 2018). The results from this thesis has provided the first empirical comparison of Msats and SNPs for any shark species and addresses some of these considerations.

Project objectives and sampling design

If a decision must be made between using a genetic (mtDNA or Msats) or genomic (SNPs) approach, it is critical to first understand the research question being addressed and if a certain type of data would best answer this (Hohenlohe et al., 2018). The work throughout this thesis shows that both genetic and genomic approaches have merit for questions of population structure and reconstruction of paternity. The assessment of population structure in *C. albimarginatus* and *C. lewini* found population genomic approaches can provide more accurate estimates of genetic statistics than traditional techniques. However the exclusive use of microsatellites successfully reconstructed paternity estimates for *S. lewini* and *C. amblyrhynchos*.

Increasing sampling effort (i.e. the number of samples collected and sampling locations) can also determine the power of an analysis and analytical methods possible for use. In both chapters 2 and 3, a lack of sampling sites across regions reduced the statistical power and capacity of the study. Several statistical analyses including nested hierarchical AMOVAS were unable to be calculated because of the opportunistic sampling design implemented. While increasing the number of nuclear loci has been found to enhance the resolution of connectivity assessments (Duchesne et al., 2000; Paetkau et al., 2004; Benestan et al., 2015) the data from this thesis also highlights the importance of a well thought out sampling design. By using a number of populations for connectivity assessment for *S. lewini* the results are more robust providing greater confidence in the interpretation. However, deeper understanding of nested population structure was unable to be acquired.

Often population genetic studies take advantage of opportunistic sampling efforts due to the challenges, expense and time required to capture sharks and rays. Moreover, sampling may be reliant on collection from fisheries as was the case for many of the PNG samples. While these collection methods made a number of samples available and subsequent population studies possible, the results from the study highlight how an opportunistic sampling design can limit downstream analyses and overall conclusions reached. This is not a novel finding and has been

previously discussed, but is nonetheless important to note (Meirmans, 2015). Preferably, sample collection with a nested design, balanced sex-ratios, high sample numbers and if possible temporal replicates would be optimal. Additionally, it would be prudent to design a sampling strategy based on simulations specific to the study species of interest. A number of genetic software packages are available to simulate realistic geographical scenarios and genetic power required to delineate structure (Hoban et al., 2012). Undertaking these simulations will be of great benefit before deciding on whether genetic or genomic approaches are required.

Analytical power to effective test hypotheses

Theoretically, increasing the number of loci can overcome methodological limitations by improving the accuracy of estimates while maintaining precision (Kohn et al., 2006; Allendorf et al., 2010). A number of studies (already discussed in previous chapters) have eluded to the power of SNPs verses Msats with 'conversion rates' (i.e. the number of loci required to obtain similar levels of informativeness) from 10:1 to 80:1 (Hohenlohe et al., 2018). In both chapters 2 and 3 we have a significant number of SNPs to Msats (upwards of 500:1) and power simulations indicated both suites of markers were able to determine genetic structure at relatively low F_{ST} values. However, the power of estimating genetic structure for SNPs was very high (consistently = 1), while Msats did show a reduced capacity when simulated F_{ST}s were very low. Consistently, sets of SNPs used in the studies were more powerful than Msats and resolution of model based and F-statistic (i.e. F_{ST}) estimations were better using SNPs.

For *C. albimarginatus*, both SNPs and Msats estimated a lack of gene flow across the Indian Ocean and putative connectivity between Australia and PNG. Conversely, in chapter 3, SNPs identified additional barriers to gene flow, unrecorded by Msats. Specifically, Msats displayed less clustering in PCA space, less Bayesian clustering and pairwise F_{ST}s were precise (similar to SNP F_{ST}s) however not accurate (non-significant). Using SNPs, additional barriers to gene flow have been identified in a range of species including polar bears (*Ursus maritimus*), honey bees (*Apis mellifera mellifera*), carp (*Carcassius carassuis*) and lobster (*Homarus americannus*) (Malenfant et al., 2015; Bernatchez, 2016; Jeffries et al., 2016; Muñoz et al., 2017). The observation of similar F_{ST} values with differing levels of significance between Msats and SNPs provide an example of the difference in accuracy and precision between markers. Despite having F_{ST} estimates of a similar magnitude (precision), SNP pairwise comparisons were often found to be statically significant while many estimates using Msats weren't (accuracy). This is likely due to the effect of using thousands of loci versus tens of loci (Allendorf et al., 2010; Benestan et al., 2015; O'Leary et al., 2018). Nonetheless, it raises the question of whether the low yet significant F_{ST} values identified using SNPs hold biological

meaning (i.e. are precise). Using additional estimates of population structure including DAPC, STRUCTURE and ADMIXTURE software has helped to resolve part of this challenge. However, the genetic subdivision identified should be treated as a hypothesis for other data sources to be tested against. Undertaking studies using methods such as genetics, parasites, life-history and morphology can help to disentangle the demographic and biological meaning of results presented here.

Mitochondrial DNA

MtDNA was used in chapters 2 & 3 and provided a number of reliable estimates for the population genetic studies, unable to be measured using nuclear DNA (Msats and SNPs). Firstly, mtDNA was used as a species identification tool to ensure all samples provided were the study species of interest (*C. albimarginatus*, *S. lewini* and *C. amblyrhynchos*). Secondly, Medium-Joining network analyses (Figures 2.2 and 3.2) were used to examine the phylogeny of clades present within our datasets. Both indicated large haplotypic distinctions between central Indo-Pacific locations and the Seychelles for *C. albimarginatus* and *S. lewini,* suggesting deep evolutionary structure and lack of connectivity between regions. Conversely, networks observed shared haplotypes across the central Indo-Pacific and Pacific Ocean, indicating historic connectivity across the regions for *C. albimarginatus* (central Indo-Pacific) and *S. lewini* (central Indo-Pacific and Pacific Ocean). These shared evolutionary signatures support colonization outwards from the central Indo-Pacific as suggested for many species of sharks (Duncan et al., 2006 b).

Overall haplotype diversities for *C. albimarginatus* and *S. lewini* were high and similar to population studies other species spanning ocean basins; e.g. basking sharks *Cetorhinus maximus*, black tip sharks *Carcharhinus limbatus*, sperm whales *Physeter macrocephalus* and Orcas *Orcinus orca* (Lyrholm et al., 1996; Hoelzel et al., 2002, 2006; Keeney et al., 2005). The identification of high haplotype and nucleotide diversities within locations such as PNG suggest at the population level genetic diversity is healthy and events of inbreeding depression are currently unlikely.

Using mtDNA the identification of Evolutionary Significant Units (ESUs) and Management Units (MUs) was possible. ESUs recognise historical population structure, identifying the evolutionary processes of genetic differentiation relevant to long-term management (Moritz, 1994). This is different from an MU or biological stock which is fundamental to short-term management and multiple MUs can occur in a single ESU (Palsbøll et al., 2007). The mtDNA results from this thesis strongly suggest the deep divergences between Seychelles and the central Indo-Pacific represent two ESU's for both *C. albimarginatus* and *S. lewini*.

Project cost and time required between markers

Another useful comparison produced from this research has been the cost and time for deploying Msats and SNPs. A number of studies have simulated and empirically tested the cost effectiveness of using genetic (Msats) or genomic (SNPs) methods for population and conservation genetic projects (Liu et al., 2005; Antao et al., 2011; Rasic et al., 2014; Kraus et al., 2015; Jeffries et al., 2016; Puckett et al., 2016; Puckett, 2017). There are discrepancies between estimations with some suggesting Msats are more cost effective (Puckett, 2017), while others proposing genome wide methods are becoming cheaper (Kraus et al., 2015). Rough cost estimates undertaken from work completed in this thesis have found microsatellites are the cheaper option per sample if primers are already characterised. For microsatellite primers already available (as in chapter 3) the cost per sample was $30.86 and cost per loci ranged from $7.72-$10.29. The price range exists because groups of either 3 or 4 loci were multiplexed together. For primers that required characterisation and optimisation (chapter 2) the cost per sample was $52.09 and cost per loci ranged from $13.02-$17.36. These estimates include library development (chapter 2), sequencing/genotyping costs, primers, PCR and clean up reagents; costs do not include DNA extraction. The SNP library development, sequencing/genotyping and bioinformatics was completed by two different companies (AGRF and DaRT) cost per sample ranged from $50.93-$56.84, while costs per loci was $1.62-$1.67. This cost per loci is a conservative estimate as it is based on the final number of SNPs used in analyses and not total raw SNPs returned from initial SNP screening. These estimates highlight that access to thousands of SNP loci is quite cost effective, however at a per sample cost microsatellites are the cheaper option if primers are available. These rough estimates support the findings of other studies including Puckett et al., (2017).

Time spent in the laboratory and at a computer (i.e. quality control and downstream filtering of loci) varied. For microsatellites all PCR and genotyping reactions were completed in-house at CSIRO Marine Facilities, Hobart. Time spent optimising PCR reactions and completing genotyping took place over months. Conversely, DNA was shipped to genomic companies for SNP discovery and apart from DNA extraction no time was spent in the laboratory for that marker type. Desktop work using microsatellite scoring software (Geneious) processed loci with screening taking a number of weeks. Quality control of SNPs and downstream filtering also took a number of weeks. In house genotyping of microsatellites took a large portion of time, however this could be reduced by outsourcing genotyping to genomic company services (at an increased cost). Certainly with regards to laboratory work, SNPs are less intensive however require a substantial amount of computational time undertaking quality control, filtering and downstream analyses.

Recommendations

When comparing the price and time spent against the output of loci per method (tens vs thousands), these findings would suggest SNPs are an incredibly cost effective, time efficient and high output method. However the ease of adding additional samples to a dataset and control of laboratory processing is costly and limited respectively. Studies in this thesis have shown both markers can provide useful assessments of population structure and paternity for shark species, however SNPs may provide more fine scale resolution if sampling design is well planned. Genomic techniques may improve the accuracy and precision of population genetic estimates especially for species with known low genome-wide F_{ST}s. Below are a number of recommendations derived from the work of this thesis for shark and ray researchers wanting to undertake population genetic studies:

- If microsatellites are already designed and samples sizes are large this will be a cheaper method for estimating population structure, however the accuracy of results may be reduced
- If microsatellites require characterisation the costs are similar to that of ddRAD SNP discovery
- SNPs are an accurate, precise and cost effective (per loci) method for population structure studies and can overcome issues of small sample size (however not poor sampling design)

5.3 Shark biology and drivers of shark movement

The movement of sharks across ocean basins has rarely been tracked using satellite tagging and is exclusive to large bodied pelagic sharks; white sharks (*Carcharodon carcharias*), basking sharks (*Cetorhinus maximus*), whale sharks (*Rhincodon typus*) and Mako sharks (*Isurus oxyrinchus*) (Eckert et al., 2001; Bonfil et al., 2005; Skomal et al., 2009; Corrigan et al., 2018). The demographic connectivity of many shark species across ocean basins is thought to be low, with few individuals migrating vast distances. Assessing the genetic connectivity of species can provide such evidence of a small number of sharks maintaining gene flow across broad ranges, for example *P. glauca* and *I. oxyrinchus* (Bailleul et al., 2018; Corrigan et al., 2018). The persistence of gene flow between regions is possible with a small number of individuals migrating and successfully breeding with individuals from neighbouring populations (Mills et al., 1996; Waples et al., 2006). Alternatively, studies have identified a lack of genetic connectivity across ocean basins for species such as the blacktip reef

shark *C. melanopterus* (Vignaud et al., 2014), grey reef shark *C. amblyrhynchos* (Momigliano et al., 2017) and tiger shark *Galeocerdo cuvier* (Holmes et al., 2017). The findings presented in chapter 2 and 3 of this thesis have updated the understanding of gene flow across ocean basins for two large bodied sharks.

Firstly, the population genetic assessment of *C. albimarginatus* (chapter 2) is the first study completed for the species and provides a basis (and successful development of novel suites of markers) for future genetic studies to be undertaken. This species has a patchy distribution throughout tropical and sub-tropical reef habitats. The lack of genetic connectivity identified between the east and west regions of the Indian Ocean means populations are most likely to be genetically discrete and an exchange of migrants is unlikely to be occurring between Pacific and Indian oceans. Secondly, the use of genome wide SNP markers have defined new barriers to gene flow for *S. lewini* throughout Pacific and Indian Oceans than was previously recorded using microsatellites (Daly-Engel et al., 2012). Genetic differentiation between central Indo-Pacific countries (Papua New Guinea, Australia, Indonesia, Philippines and Taiwan) and ocean basin neighbours Seychelles, Hawaii and Gulf of California provides evidence; *S. lewini* are not often traversing vast oceanic distances.

Large bodied sharks such as *C. albimarginatus* and *S. lewini* display cryptic use of reef habitat (i.e. do not inhabit continuously) similar to *G. cuvier*, *C. leucas*, *C. obscurus and Negaprion acutidens* (Hearn et al., 2010; Meyer et al., 2010; Papastamatiou et al., 2013; Espinoza et al., 2014 a, 2015 b; Ketchum et al., 2014). As adults, both species are often found inhabiting pelagic waters off continental shelves and sea mounts (Last & Stevens 2009). However, contrary to *C. albimarginatus*, tagging studies have shown *S. lewini* to disperse and spend time in coastal regions (a behaviour attributed to pupping in sheltered coastal refuges) and have been found in pelagic waters, captured in oceanic fisheries (Kohler et al., 2001; Hoyos-Padilla et al., 2014; Yates et al., 2015). While both species generally spend less time on coral reefs than smaller bodied “true reef sharks” (e.g. *C. amblyrhynchos*, *C. melanopterus*, *Triaenodon obesus*) (Frisch et al., 2016) they do have a reliance on reef ecosystems possibly for food, refuge and locating breeding partners (Espinoza et al., 2015 a; Bond et al., 2015). It is likely these fundamental requirements reduce trans-oceanic movements.

The diet of scalloped hammerheads is diverse and often dependent on what species are available within its residential habitat. Juveniles in Hawaii are known to feed on benthic shrimp and gobies (Bush, 2003), while in Mexico are found to feed on combination of cephalopods, crustaceans and a number of carangid fishes (Torres-Rojas et al., 2010). In South Africa, *S. lewini* are observed to have ontogenetic shifts with juveniles feeding on coastal cephalopods and larger animals feeding on more pelagic cephalopods (Smale et al., 1998). Dietary analysis for *C. albimarginatus* is less known

than that of *S. lewini*; only two studies have reported dietary information finding a combination of fish, cephalopods and invertebrates present in stomachs (Cortés, 1999). Clearly, *S. lewini* have a diverse diet with foraging occurring in varied marine habitats (coastal, reef, pelagic) while more dietary studies are required to better understand *C. albimarginatus* foraging. Tagging studies have shown individual *C. albimarginatus* and *S. lewini* often maintain a level of residency within interconnected reef systems. For *S. lewini*, tracking has observed adults leaving reef systems to enter pelagic waters, a movement attributed with foraging (Ketchum et al., 2014). Often, individuals were observed to visit neighbouring reefs or return to tagged reefs after these excursions. Given *S. lewini* feed on diverse prey items it would seem their residency to reef systems is not driven by diet. Tagging of *C. albimarginatus* undertaken by Espinoza et al., (2015 a) described the residency of *C. albimargtinus* within the Great Barrier Reef to be driven by foraging, reproduction and predator avoidance. The kin ship analysis completed for *C. albimarginatus* eludes to possible familial residency for a (6-7 year old) sibling pair near Manus Island, PNG. More detailed knowledge is required (including robust dietary analysis for *C. albimarginatus*) to fully understand why trans-oceanic gene flow appears to be limited and why residency within connected reef systems is observed for *C. albimarginatus* and *S. lewini*.

In contrast to a lack of trans-oceanic connectivity across the Pacific and Indian Ocean basins, gene flow was identified between countries within the central Indo-Pacific whose continental shelves provide continuous habitat (PNG, Australia, Indonesia, Philippines and Taiwan). This thesis has detailed possible connectivity between PNG and Australia, noting more sampling across the range is required to fully understand the extent of gene flow (especially for *C. albimarginatus*). Connectivity identified for *S. lewini* was directly correlated with geographic distance and it seems likely individuals are dispersing broadly throughout the Indo-Pacific. A number of studies have suggested the prevalence of dispersal in reef sharks is influenced by the degree of reef isolation (Espinoza et al., 2015 b; Heupel et al., 2018 b). Telemetry studies on *C. amblyrhynchos*, *C. albimarginatus* and *C. melanopterus* in the interconnected reef system of the GBR all found individuals more likely to undertake regular excursions away from their tagged reef (Heupel et al., 2010; Chin et al., 2013; Espinoza et al., 2015 a). However, studies on same species *C. amblyrhynchos* and *C. melanopterus* at the isolated reefs of Palmyra Atoll found movement is limited (Papastamatiou et al., 2010; White et al., 2017). Certainly, the Indo-Pacific and study area of chapters 2 and 3 provides closely located reef habitat to promote movement. Reefs in PNG are closely linked to the continental shelf of Australia and the complex reefs of the GBR. Additionally, shallow (< 200m deep) water and suitable reef structures exists between PNG, Australia and Indonesia.

As suggested in chapters 2 and 3 movement between connected reefs could be driven by reproductive requirements. Observed dispersal of reef sharks is not uniform and both size and sex play a role in an individual's propensity to disperse (Espinoza et al., 2015 b). A number of reef associated species have ontogenetic changes as they move through size classes, in general smaller individuals are more resident and larger individuals more likely to disperse (Wetherbee et al., 1996; Simpfendorfer et al., 2001; Heupel et al., 2018 b). The data from this thesis would certainly support such observations since gene flow is apparent between PNG and neighbouring nations. This is important given species that move large distances (e.g. between PNG and Australia) can serve as mobile links, providing energy transfer among ecosystems (Nyström et al., 2001; Heupel et al., 2015).

Another significant finding in this thesis was the occurrence of multiple paternity in *S. lewini* and *C. amblyrhynchos* from PNG. As previously discussed the mating strategies of sharks are diverse and not well documented for most shark species (Byrne et al., 2012). Therefore, when samples from gravid females are made available studies should attempt to understand breeding strategies. The identification of polyandrous behaviours leading to multiple paternity provides a number of benefits including increasing the likelihood of producing offspring (i.e. fitness), increasing genetic variation, counteracting issues of inbreeding and increasing the effective population size (all discussed in more detail in chapter 4) (Sugg et al., 1994; Chapman et al., 2004; Neff et al., 2005; Daly-Engel et al., 2006). Additional findings of a correlation between adult size and litter size for *S. lewini* is of great interest however requires further robust sampling. Observations of such effects combined with genetic diversity estimates and information on size and sex-specific landings for the species can help identify if, and where, populations of these species are at risk of reduced genetic diversity and potential population declines. The prevalence of multiple paternity in combination with high genetic diversity calculated for *S. lewini* in PNG (chapter 3) indicate the population is currently not at risk of inbreeding depression.

5.4 Management considerations

The research in this thesis has shown that biological stocks of the two species most likely extend across the economic zones of Papua New Guinea and Australia, meaning both countries are likely to be exploiting the same resource. Maintaining genetic connectivity is important in long-term conservation planning and requires on-going monitoring (Wallace et al., 2010). The region between Australia and PNG not only represents two nations EEZ's but also three marine management areas (in Australian waters); the GBR, Torres Strait and Coral Sea. These areas are managed under a

combination of national and state governments adding to the complexity. In both Australia and PNG traditional owners and indigenous tribes have concomitant defined boundaries along with differing social, economic and cultural values (Nursey-Bray, 2011; White et al., 2018). In order to accomplish cross-jurisdictional management, coordination between varying governmental, corporate and indigenous stakeholder groups are required to understand the complex use of recourses. This section does not mean to recommend best management strategies for shark resources between PNG and Australia as that is outside the scope of this thesis. Instead, the results presented here highlight the complex management and stakeholder engagements required in light of the observed genetic connectivity of *C. albimarginatus* and *S. lewini* between PNG and Australia.

It is likely shark catches in PNG have reduced since the closure of the target shark and ray fishery in 2014. However, tuna longline vessels and artisanal fishing is still landing a large number of sharks (White et al., 2018). The species discussed in this thesis; *C. amblyrhynchos*, *C. albimarginatus* and *S. lewini* are listed as Near Threatened, Vulnerable and Endangered respectively under the IUCN Red List. This listing also describes *C. albimarginatus* populations as in decline and the other species as unknown. If adults are persistently removed from PNG waters, this may likely reduce recruitment for the region. While the work from this thesis has identified genetic connectivity between PNG and Australia, it is unknown how much movement is occurring temporally. Until demographic migration rates are measured it is unknown if connectivity is enough to replenish fished populations. As suggested by Smart et al., (2017 a) exclusively removing juvenile sharks may help to better manage the stocks and increase numbers of large breeding adults in the region. The removal of sharks from an ecosystem can have varying and detrimental impacts as their roles of apex and meso-predators often maintain healthy ecosystems (Robbins, 2006; Heupel et al., 2019). As defined by Simpfendorfer & Dulvy 2017, sustainable shark fishing is possible when the fisheries authority has (i) information on demographic population growth, (ii) precautionary science-based catch limits, (iii) where applicable international treaties (i.e. CMS, CITES) for monitoring of trade, (iv) support from developed countries to aid in capacity building of sustainable fisheries and (v) traceability of products entering the market (Simpfendorfer & Dulvy 2017). The PNG National Fishing Authority are working towards having such information and undertaking capacity building activities (White et al., 2018). It is hoped with future research in the PNG area, utilization of shark and ray resources can be a viable, long-term sustainable option for the PNG people.

5.5 Future Directions

An objective of this thesis was to use genetic and genomic methods to estimate the population structure of shark species between PNG and neighbouring nations. The results from chapters 2 and 3 suggest genetic connectivity is occurring between nations. It is crucial that future work attempts to test the baseline hypothesis generated from genetic results to estimate the demographic importance of this observation. Combining genetic findings with techniques such as acoustic telemetry (Corrigan et al., 2018) and parasites (Welch et al., 2015) will be of great interest. Understanding how species move across environments is essential for identifying critical habitats or corridors that may be important for maintaining population connectivity (Pascual-Hortal et al., 2006; Fletcher et al., 2011); making accurate estimations of future population resilience (Olds et al., 2012); and developing management strategies to ensure long-term conservation (Bond et al., 2012; Knip et al., 2012).

It will be of great interest to understand if adaptive or selective pressures affect the distribution of the species studied in this thesis. Currently, there are no well annotated reference genomes for any shark species, thereby making the estimation of candidate/adaptive loci superfluous. Recent studies have attempted to use candidate loci for shark population assignment however the final conclusions are speculative (Junge et al., 2019). Additionally, in order to understand if selection can be used to predict dispersal and/or residency, comprehensive environmental data collection (abiotic and biotic measures) is required. Understanding adaptation in sharks may provide further insight into why sharks decide to disperse or remain resident and ultimately aid in future connectivity assessments.

The study in chapter 2 was limited by its' sampling design (i.e. limited collection locations) and identifies genetic connectivity for a small part of *C. albimarginatus's* range within the Indo-Pacific. As a shark species that had not previously been examined genetically, more population genetic research is required to understand connectivity at a global scale. This includes collecting samples from south of the Seychelles; Madagascar and the east African Coast, as well as through the Gulf of Mexico, Central America the west coast of the USA and Hawaii to see if any exchange is occurring. Samples from Philippines, Taiwan, India and Indonesia will be of interest given the fishing pressure on sharks and rays is significant in those regions. All datasets used in the analyses presented here are available to researchers and will provide a significant body of work for future research projects on these species.

The work presented in this thesis is a snapshot of the genetic connectivity for two species of shark often captured in numerous fisheries. Given maintenance of connectivity between locations requires long-term management, it is recommended curating tissue samples from these species be undertaken for future assessments of possible temporal changes.

The findings also suggests the potential underestimation of population structure using microsatellites. Each marker deployed in this research detected similar trends of population connectivity, however quantitative estimates were slightly different. The increased power afforded by using thousands of SNPs enabled the detection of additional barriers to gene flow unresolved using microsatellites. In chapter 3, SNP results more closely matched mtDNA than nuclear DNA (Msats). Due to the uneven collection of males and females per location in our study, sex-biased measurements were unable to be calculated. Future studies using a combination of mtDNA and nuclear DNA should account for this in sampling designs; collecting even numbers of adult males and females.

The work produced throughout this thesis has provided a number of novel findings addressing current knowledge gaps for sharks and the study of population genetics including; the first assessment of the genetic population structure of *C. albimarginatus* and two case studies empirically comparing microsatellite and SNP markers for population assignment of sharks. These findings are used to directly estimate the genetic stock structure of the two shark species and results provide a road map for marker choice for future population genetic studies.

A) Appendix I. Supplementary material from chapter 2; Novel multi-marker comparisons address the genetic population structure of silvertip sharks (*Carcharhinus albimarginatus*)

a) Tables and Figures

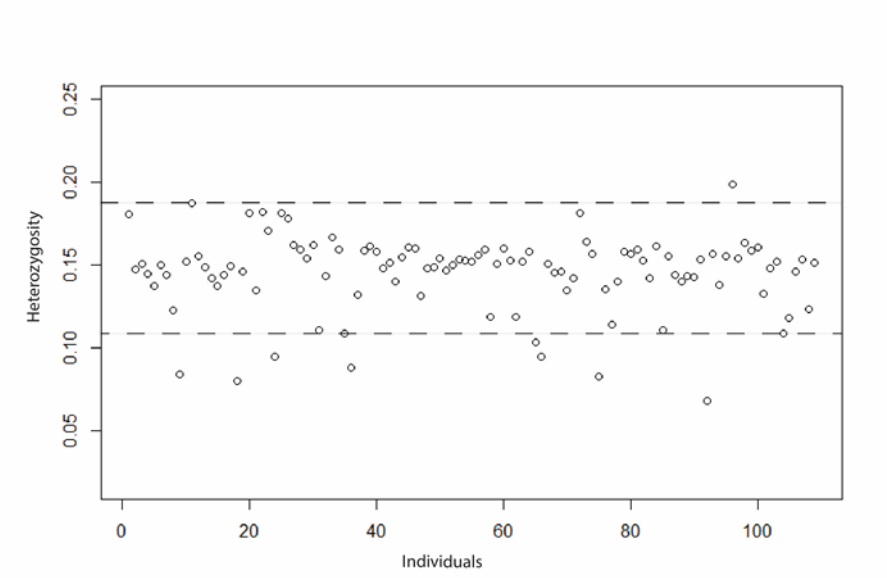
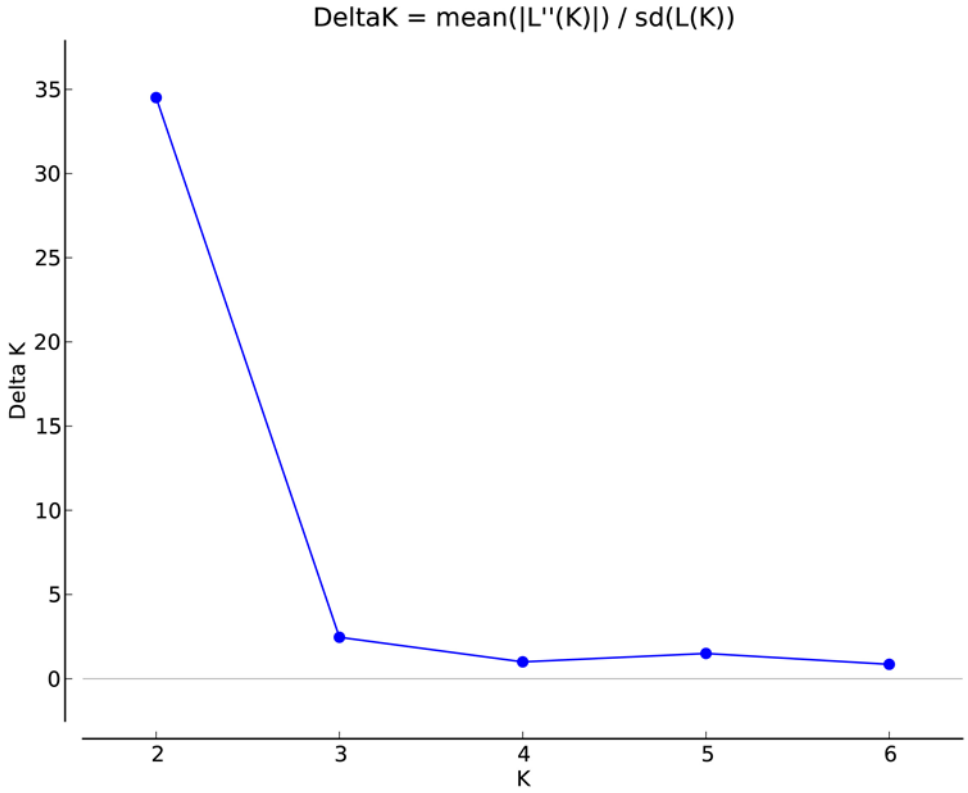


Figure A.1. Average heterozygosity of SNP loci per individual during filtering process (not final SNP set of 6,461 SNPs). Dashed lines represent cut off range (< 0.11 and > 0.18) in the SNP filtering process. Heterozygosity was filtered to remove potential individuals of poor DNA quality or sample contamination. Thresholds were selected to remove individuals outside average range for the SNP dataset (0.11-0.18).



K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	8	-2688.81	0.155265	—	—	—
2	8	-2642.16	3.494869	46.65	120.6125	34.51131
3	8	-2716.13	21.58583	-73.9625	53.1125	2.460527
4	8	-2736.98	54.98113	-20.85	54.9625	0.999661
5	8	-2702.86	24.39865	34.1125	36.575	1.499058
6	8	-2705.33	28.72648	-2.4625	24.525	0.853742
7	8	-2683.26	13.01581	22.0625	—	—

Figure A.2. Outputs from microsatellite STRUCTURE analysis showing Evanno's Delta K value (above), a method based on the rate of change in log probability of data and Evanno table output for K = 1-7 (below).

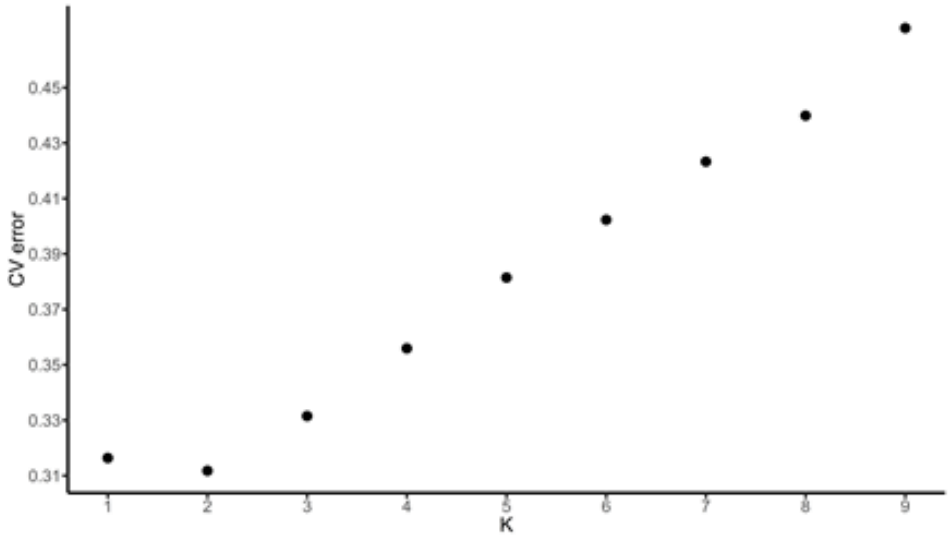


Figure A.3. Outputs from SNP ADMIXTURE analysis showing CV error values for scenarios of K = 1-9. Note K = 2 has the lowest CV value.

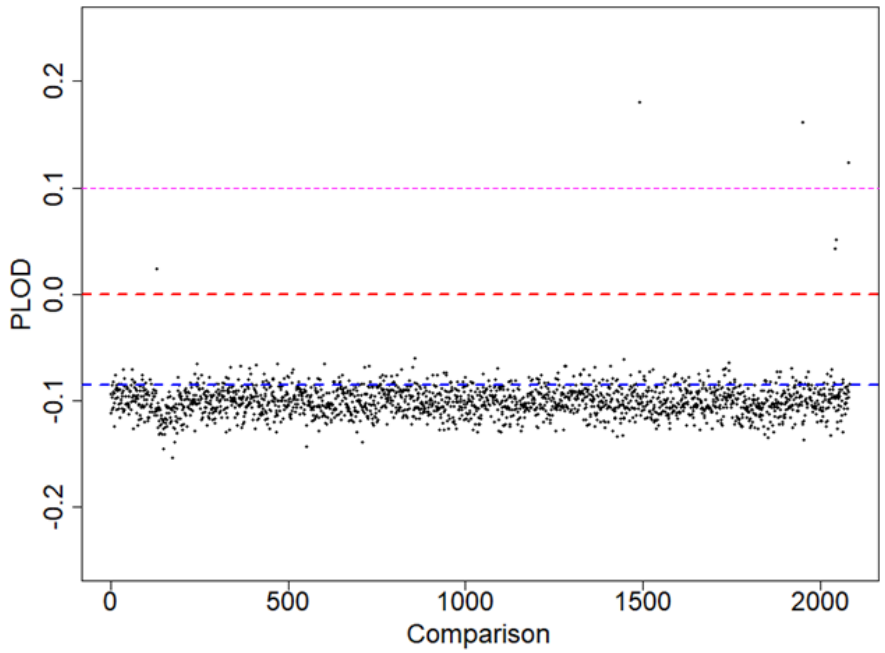


Figure A.4. Output from kin inference using method described in Hillary *et al* (2018). Each point is a comparison of SNP genotypes of two individuals plotted against the PLOD score. The blue and magenta lines denote the expected values for unrelated and full-sibling pairs (UP and FSP) respectively. The red line is representative of the cutoff, where anything above is estimated either FSP/POP.

Locus	Primer sequence (5'-3')	Repeat motif	Size range (bp)	T_A (°C)	N_A	PIC	GenBank Accession number
[1]ALS1	F: GGTTGGTCTCCAGAGTTGGG[PET]	(GA)	268-322	55	30	0.906	KY996371
	R: GTCAACATGATGTGCCAGCG						
[1]ALS2	F: AAGCAATGGACTGTGGCGAT[VIC]	(GA)	276-288	55	7	0.667	KY996372
	R: GGCGAACTTCACATCTTGCC						
[4]ALS4	F: AGGCTGGATGTAGCAAGCAA[VIC]	(TG)	288-300	50	7	0.747	KY996382
	R: TTACATCCCGGAGTGGACCA						
[1]ALS6	F: GAAGCGATGAGGGAGGCC[FAM]	(TG)	284-294	55	6	0.689	KY996373
	R: GGACAGTCCACCATTCACCC						
[2]ALS7	F: CGTAGGCTCGCTGACATCAT[NED]	(GA)	223-231	55	5	0.299	KY996376
	R: TAGGTGCTTGAAGGCCACTG						
[3]ALS9	F: CAGCTCTCCCTCCACAATCG[FAM]	(AG)	232-234	50	2	0.058	KY996379
	R: TTCCTTTCAATCGGAGGCCC						
[2]ALS11	F:GGGCTTCTTGGACACTTTGTG[FAM]	(TG)	296-338	55	2	0.009	KY996377
	R: GCAGTGCTTACCAACATGCC						
[4]ALS14	F: TTCTCTGTTCCTGTTGGCCC[FAM]	(AC)	235-276	50	2	0.519	KY996381
	R: TGAGCTATCCCAGTCCCTCC						
[2]ALS23	F: TCATAGTGGGCAGGGATGGA[VIC]	(GA)	248-274	55	3	0.515	KY996375
	R: TGGTTTGGCCTCAGCTCATT						
[3]ALS42	F: TGCCGTACTGAGTAGATCCCT[NED]	(CCCT)	240-260	50	5	0.684	KY996378
	R: GGGAGCCAGGACCCAGATTA						
[3]ALS51	F: GCATCGAGGGATCATATTGACA[PET]	(AGG)	289-292	50	2	0.009	KY996380
	R: GACTTTGGTGCAGAGGGTCA						
[1]ALS52	F: CCAGTGCTTACTTTGTGCTGT[NED]	(TTG)	237-267	55	8	0.467	KY996374
	R: AGGAAGCCGTGAATGACAAA						

Table A.1. Novel microsatellite loci for *C. albimarginatus* isolated in this study

Annealing temperature (T_A), sample size (*n*), number of alleles (N_A) and polymorphic information content (PIC). Superscript represents multiplex reaction (1, 2, 3 or 4)

Locations	Parameter	ALS1	ALS2	ALS4	ALS6	ALS7	ALS9	ALS11	ALS14	ALS23	ALS42	ALS51	ALS52
Seychelles (n=30)													
	n	29	29	30	29	29	29	29	29	29	30	30	29
	N_A	17	4	4	4	2	1	1	1	3	4	1	5
	H_O	0.862	0.552	0.733	0.793	0.200	0.000	0.000	0.000	0.433	0.724	0.000	0.414
	H_E	0.831	0.515	0.712	0.661	0.180	0.000	0.000	0.000	0.399	0.681	0.000	0.407
	F_{IS}	-0.04	-0.07	-0.03	-0.20	-0.11	NA	NA	NA	-0.08	-0.06	NA	-0.02
	HWE_p	0.684	0.139	0.894	0.230	1.000	1.000	1.000	1.000	0.826	0.572	1.000	0.659
Papua New Guinea (n=64)													
	n	62	62	64	62	55	64	64	64	64	64	58	58
	N_A	22	7	7	6	5	2	2	2	3	5	2	7
	H_O	0.855	0.694	0.828	0.710	0.345	0.078	0.016	0.063	0.641	0.656	0.000	0.466
	H_E	0.904	0.741	0.804	0.739	0.409	0.075	0.016	0.061	0.587	0.746	0.034	0.479
	F_{IS}	0.05	0.06	-0.03	0.04	0.16	-0.04	-0.01	-0.03	-0.09	0.12	1.00	0.03
	HWE_p	**0.045**	0.129	0.062	0.182	0.433	1.000	1.000	1.000	0.818	0.068	**0.009**	0.800
East Australia (n=23)													
	n	20	22	23	22	21	23	20	23	23	23	21	16
	N_A	18	6	5	5	3	2	1	2	3	5	1	5
	H_O	0.900	0.818	0.783	0.818	0.143	0.043	0.000	0.043	0.522	0.696	0.000	0.438
	H_E	0.916	0.723	0.765	0.727	0.353	0.043	0.000	0.043	0.589	0.733	0.000	0.594
	F_{IS}	0.02	-0.13	-0.02	-0.13	0.59	-0.02	NA	-0.02	0.11	0.05	NA	0.26
	HWE_p	0.180	0.217	0.199	0.936	**0.004**	1.000	1.000	1.000	0.385	**0.006**	1.000	0.311

Table A.2. Summary statistics for microsatellite loci per population.

The table describes the following parameters per microsatellite loci for each location; Number of alleles (NA), observed (HO) and expected (HE) heterozygosity, inbreeding coefficient (FIS), Hardy-Weinberg Equilibrium p value (HWEP). Bold HWEp values are consider statistically significant ($p < 0.05$).

Table A.3. Allele frequencies per location, per locus for 12 microsatellite loci. See Table A.2 for sample sizes per locus per collection location.

ALS 1	Seychelles	Papua New Guinea	East Australia
268	0.350	0.211	0.109
270	0.050	0.047	0.022
272	0.083	0.023	0.130
274	0.050	0.070	0.022
276	0.017	0.055	0.109
278	0.083	0.117	0.087
280	-	0.078	0.043
282	-	0.070	0.043
284	-	0.063	0.022
286	0.017	0.039	0.043
288	-	0.008	0.043
290	-	0.016	-
292	0.100	-	-
294	-	-	0.043
296	0.033	0.008	-
298	-	-	0.043
300	0.033	-	-
302	-	0.047	0.022
304	0.017	0.016	-
306	-	0.016	0.022
308	-	-	0.022
310	0.033	0.016	0.022
312	0.017	-	-
316	0.017	-	-
318	0.033	0.023	-
320	0.017	0.016	-
322	0.017	0.008	-
326	-	0.008	-
330	-	0.016	-
332	-	-	0.022
ALS 2	Seychelles	Papua New Guinea	East Australia
276	-	0.086	0.174
278	0.600	0.281	0.283
280	0.017	0.117	0.065
282	0.050	0.086	-
284	0.300	0.367	0.370
286	-	0.023	0.043
288	-	0.008	0.022
ALS 4	Seychelles	Papua New Guinea	East Australia
288	-	0.055	-
290	0.383	0.242	0.348
292	0.217	0.133	0.130

294	0.117	0.141	0.196
296	0.283	0.297	0.217
298	-	0.078	0.109
300	-	0.055	-
ALS 6	Seychelles	Papua New Guinea	East Australia
284	-	0.047	-
286	0.117	0.133	0.130
288	0.083	0.086	0.109
290	0.417	0.320	0.370
292	-	0.047	0.065
294	0.350	0.336	0.283
ALS 7	Seychelles	Papua New Guinea	East Australia
223	-	0.008	0.043
225	0.100	0.188	0.152
227	-	0.023	-
229	0.900	0.633	0.717
231	-	0.008	-
ALS 9	Seychelles	Papua New Guinea	East Australia
232	0.967	0.961	0.978
234	-	0.039	0.022
ALS 11	Seychelles	Papua New Guinea	East Australia
298	1.000	0.992	1.000
300	-	0.008	-
ALS 14	Seychelles	Papua New Guinea	East Australia
263	0.967	0.969	0.978
265	-	0.031	0.022
ALS 23	Seychelles	Papua New Guinea	East Australia
248	0.067	0.352	0.478
252	0.750	0.523	0.413
254	0.183	0.125	0.109
ALS 42	Seychelles	Papua New Guinea	East Australia
240	-	0.031	0.087
244	0.367	0.336	0.348
248	0.317	0.266	0.261
252	0.250	0.219	0.261
260	0.033	0.148	0.043
ALS 51	Seychelles	Papua New Guinea	East Australia
289	-	0.016	-
292	0.933	0.891	0.957
ALS 52	Seychelles	Papua New Guinea	East Australia
237	0.083	0.008	0.022
240	-	0.008	-
243	0.733	0.625	0.391

246	0.083	0.180	0.196
249	-	0.070	0.065
252	-	0.008	-
255	0.017	-	0.022
258	0.050	0.008	-

Filtering step	SNPs remaining	Individuals remaining
Initial SNPs	717,800	
Initial Individuals		n = 146
Remove linked SNPs	412,771	
Retain call rate per SNP (> 0.7)	88,207	
Remove monomorphic SNPs	57,400	
Retain call rate per ind. (> 0.80)		n = 109
Remove monomorphic SNPs	56,171	
Remove SNPs with Minor Allele Frequency (< 0.02)	32,942	
Retain SNPs with Heterozygosity per individual (between 0.11-0.18)		n = 92
Remove SNPs with HWE (< 0.05)	29,549	
Remove monomorphic SNPs	29,212	
Retain call rate per SNP (= 1.0)	6,461	
TOTAL	6,461	n = 92

Table A.4. Filtering process for SNPs identified for *C. albimarginatus*.

Table A.5. Accompanying metadata for individuals identified as either Full Siblings (FS) or Parent-Offspring Pairs (POP) using the kinship inference method from Hillary *et al*., (2018).

Tag ID	Kin relationship	PLOD	Sex	Total Length (cm)	Age (years)	Location	Latitude	Longitude	Date collected
J2648	POP	0.043	F	161	11-12	Wheeler Reef, Australia	-18.80432	147.52258	23/04/2013
R1656			F	77	1-2	Wheeler Reef, Australia	-18.80432	147.52258	23/04/2013
J2648	POP	0.051	F	161	11-12	Wheeler Reef, Australia	-18.80432	147.52258	23/04/2013
R6298			M	80	1-2	Wheeler Reef, Australia	-18.80292	147.52162	22/04/2013
R1656	FSP	0.124	F	77	1-2	Wheeler Reef, Australia	-18.80432	147.52258	23/04/2013
R6298			M	80	1-2	Wheeler Reef, Australia	-18.80292	147.52162	22/04/2013
50115	FSP	0.181	M	124	6-7	Manus Island, PNG	-2.61304	146.44194	20/05/2014
50151			F	127	6-7	Manus Island, PNG	-2.61304	146.44194	31/05/2014
10177	POP	0.024	F	218	16-18	Sudest Island, PNG	-11.74051	154.09861	12/06/2014
10219			F	137	7-8	Sudest Island , PNG	-11.74051	154.09861	20/06/2014
J2617	FSP/POP?	0.162	F	113	5-6	Wheeler Reef, Australia	-18.80432	147.52258	23/04/2013
J2636			F	12.9	*	Wheeler Reef, Australia	-18.80292	147.52162	22/04/2013

Asterisk indicates likely incorrect Total Length measurement since *C. albimarginatus* pups are born ~72cm (Smart et al., 2017 a), therefore likely relationship (ie. POP or FS) cannot be determined (?). Age estimates are based on Total Length (cm) and sex specific growth curves calculated in Smart et al., (2017 b).

Table A.6. Results of power analysis conducted in POWSIM for microsatellites and SNPs.

12 Microsatellites				6,461 SNPs			
F_{ST}	t	N*e*	Power	F_{ST}	t	N*e*	Power
0.05	100	1000	1.000	0.05	100	1000	1
0.02	50	1000	1.000	0.02	50	1000	1
0.01	20	1000	0.985	0.01	20	1000	1
0.004	10	1000	0.669	0.004	10	1000	1
0.001	2	1000	0.102	0.001	2	1000	1
0.05	200	2000	1	0.05	200	2000	1
0.02	100	2000	1	0.02	100	2000	1
0.01	40	2000	0.989	0.01	40	2000	1
0.004	16	2000	0.513	0.004	16	2000	1
0.001	5	2000	0.129	0.001	5	2000	1

Time in generations (t), effective population size of subpopulations (N*e*).

Table A.7. Location data for known collection points of individuals used in this study.

Sample ID	Lat	Long	Reef/Island/Sea	Location
J2686	-18.7430	147.2600	Keeper	East Australia
J2622	-18.5238	147.3928	Glow	East Australia
R5557	-18.5188	147.3851	Glow	East Australia
J2633	-18.4677	146.8587	Rib	East Australia
J2669	-18.7445	147.2524	Keeper	East Australia
J2674	-18.6322	147.0156	Brewer	East Australia
J2633	-18.6839	147.1022	Lodestone	East Australia
J2630	-18.6223	147.2871	Helix	East Australia
J2636	-18.8029	147.5216	Wheeler	East Australia
R5561	-18.8029	147.5216	Wheeler	East Australia
J2614	-18.8029	147.5216	Wheeler	East Australia
J2627	-18.8029	147.5216	Wheeler	East Australia
R1632	-18.8029	147.5216	Wheeler	East Australia
R5590	-18.8029	147.5216	Wheeler	East Australia
J2618	-18.8029	147.5216	Wheeler	East Australia
J2649	-18.8029	147.5216	Wheeler	East Australia
R6298	-18.8029	147.5216	Wheeler	East Australia
R5582	-18.8029	147.5216	Wheeler	East Australia
J2617	-18.8043	147.5226	Wheeler	East Australia
J2623	-18.8043	147.5226	Wheeler	East Australia
R1656	-18.8043	147.5226	Wheeler	East Australia
J2648	-18.8043	147.5226	Wheeler	East Australia
J2816	-18.7381	147.2643	Keeper	East Australia
J2815	-18.6220	147.2920	Helix	East Australia
J2972	-18.5131	147.3903	Glow	East Australia
R6204	-18.5131	147.3903	Glow	East Australia
J3014	-18.5298	147.3855	Glow	East Australia
J3019	-18.6240	147.2895	Lodestone	East Australia
J3105	-18.6216	147.3026	Helix	East Australia
J3108	-18.6994	147.0905	Lodestone	East Australia
J3102	-18.7586	147.2574	Keeper	East Australia
J3123	-18.7586	147.2574	Keeper	East Australia
J3136	-18.7586	147.2574	Keeper	East Australia
C. albi 1	-4.7412	55.4297	Mahé	Seychelles
C. albi 2	-4.7412	55.4297	Mahé	Seychelles
C. albi 3	-4.7412	55.4297	Mahé	Seychelles
C. albi 4	-4.7412	55.4297	Mahé	Seychelles
C. albi 5	-4.7412	55.4297	Mahé	Seychelles
C. albi 6	-4.7412	55.4297	Mahé	Seychelles
C. albi 7	-4.7412	55.4297	Mahé	Seychelles
C. albi 8	-4.7412	55.4297	Mahé	Seychelles
C. albi 9	-4.7412	55.4297	Mahé	Seychelles
C. albi 10	-4.7412	55.4297	Mahé	Seychelles
C. albi 11	-4.7412	55.4297	Mahé	Seychelles

C. albi 12	-4.7412	55.4297	Mahé	Seychelles
C. albi 13	-4.7412	55.4297	Mahé	Seychelles
C. albi 14	-4.7412	55.4297	Mahé	Seychelles
C. albi 15	-4.7412	55.4297	Mahé	Seychelles
C. albi 16	-4.7412	55.4297	Mahé	Seychelles
C. albi 17	-4.7412	55.4297	Mahé	Seychelles
C. albi 18	-4.7412	55.4297	Mahé	Seychelles
C. albi 19	-4.7412	55.4297	Mahé	Seychelles
C. albi 20	-4.7412	55.4297	Mahé	Seychelles
C. albi 21	-4.7412	55.4297	Mahé	Seychelles
C. albi 22	-4.7412	55.4297	Mahé	Seychelles
C. albi 23	-4.7412	55.4297	Mahé	Seychelles
C. albi 24	-4.7412	55.4297	Mahé	Seychelles
C. albi 25	-4.7412	55.4297	Mahé	Seychelles
C. albi 26	-4.7412	55.4297	Mahé	Seychelles
C. albi 27	-4.7412	55.4297	Mahé	Seychelles
C. albi 28	-4.7412	55.4297	Mahé	Seychelles
C. albi 29	-4.7412	55.4297	Mahé	Seychelles
C. albi 30	-4.7412	55.4297	Mahé	Seychelles
C. albi 31	-4.7412	55.4297	Mahé	Seychelles
PNG010036	-2.2984	149.8775	Bismarck Sea	PNG
PNG010074	-2.9506	146.7702	Bismarck Sea	PNG
PNG010079	-2.8470	146.6702	Bismarck Sea	PNG
PNG010126	-2.2340	150.8616	Bismarck Sea	PNG
PNG050141	-2.8344	146.5475	Bismarck Sea	PNG
PNG050034	-2.1376	149.8211	Bismarck Sea	PNG
PNG050128	-1.3764	149.1944	Bismarck Sea	PNG
PNG050137	-2.4199	146.1661	Bismarck Sea	PNG
PNG050115	-1.8319	145.2628	Bismarck Sea	PNG
PNG050122	-2.4862	146.2253	Bismarck Sea	PNG
PNG050150	-2.8344	146.5477	Bismarck Sea	PNG
PNG050151	-2.6130	146.4420	Bismarck Sea	PNG
PNG050210	-2.6130	146.4420	Bismarck Sea	PNG
PNG050213	-2.1542	150.0844	Bismarck Sea	PNG
PNG050214	-1.8862	150.0737	Bismarck Sea	PNG
PNG050217	-1.5151	149.4378	Bismarck Sea	PNG
PNG050218	-1.3764	149.1945	Bismarck Sea	PNG
PNG050219	-1.3764	149.1945	Bismarck Sea	PNG
PNG060018	-2.3165	149.8713	Bismarck Sea	PNG
PNG060019	-1.8110	143.8187	Bismarck Sea	PNG
PNG060058	-1.8110	143.8187	Bismarck Sea	PNG
PNG060060	-1.8351	144.1117	Bismarck Sea	PNG
PNG060061	-3.1043	142.6693	Bismarck Sea	PNG
PNG060062	-3.1043	142.6693	Bismarck Sea	PNG
PNG070055	-3.1043	142.6693	Bismarck Sea	PNG
PNG090521	-2.4268	145.9958	Bismarck Sea	PNG

PNG010177	-11.7405	154.0986	Solomon Sea	PNG
PNG030100	-5.8309	154.4286	Solomon Sea	PNG
PNG030170	-3.8053	153.2653	Solomon Sea	PNG
PNG030200	-5.8309	153.2653	Solomon Sea	PNG
PNG030239	-5.1475	154.3548	Solomon Sea	PNG
PNG040323	-11.0094	155.3846	Solomon Sea	PNG
PNG040324	-11.0094	155.3846	Solomon Sea	PNG
PNG040336	-11.0428	155.3846	Solomon Sea	PNG
PNG040353	-11.0367	155.3372	Solomon Sea	PNG
PNG040354	-11.0367	155.3372	Solomon Sea	PNG
PNG040355	-11.0367	155.3372	Solomon Sea	PNG

PNG- Total per region	*n*
Above Bismarck Archipelago	27
Below Bismarck Archipelago	10
Unknown location	45

Unknown location- Samples were collected on observer vessels and fin markets, some collection locations were unable to be reported. While exact locations can't be reported it's highly likely artisanal fishers have not collected sharks outside of PNG's EEZ (see Appleyard et al., 2017).

b) Supplementary Materials

Microsatellite primer design, characterisation and optimisation

DNA was extracted using the Wizard© SV Genomic DNA Purification system (Promega); tissue extractions were undertaken using SV minicolumns following modifications to the manufacturer's instructions (i.e. overnight tissue digestion; reduction of supernatant for DNA elution and increased DNA elution time). A single sample of purified *C. albimarginatus* DNA (130 ng/µl), representing the Indo-Pacific region, was sent to the AGRF for library preparation and next generation sequencing on the Illumina®MiSeq (Illumina) (2 × 250bp end reads) with base calling undertaken using Real Time Analysis v1.18.54. The Illumina bcl2fastq 2.17.1.14 pipeline was used to generate the sequence data, with the FASTAQ sequences stitched using PEAR assembler (Zhang et al., 2014).

Shotgun sequencing resulted in 20 469 712 paired-end reads (10.23Gb). Microsatellite detection of the sequenced sample was performed using QDD (v 3.1.2) (Meglecz et al., 2014) http://gsite.univ-provence.fr/gsite/Local/egee/dir/meglecz/QDD.html) and Primer3 (Rozen et al., 2000) was used to design primers for the detected microsatellites. Following QDD detection, 229 348 putative microsatellite loci were detected. The following filters were applied to further screen the loci (according to Meglecz et al., (2014); a) primer alignment score between 1-2.75, b) minimum primer target distance between 80-147 base pairs (bp), c) length of PCR product < 305 bp, d) pure microsatellites, (repeats > 6), e) no homopolymers, f) no micro and nanosatellites in the flanking regions, g) no compound microsatellites, h) a Primer3 penalty value of < 3. Filtering resulted in 30 loci being selected for initial PCR optimization using unlabelled primers.

PCR amplification and optimisation was tested using DNA from eight *C. albimarginatus* individuals (from across different spatial locations). PCR amplifications conditions consisted of 1× GoTaq© Colourless Master Mix (Promega), 1 μL Bovine Serum Albumin (Promega,), 0.2 μM of each individual F and R primer, and 0.8 ng/µl DNA in a 25 μL reaction volume. Thermal cycling (in an Eppendorf Mastercycler®, Eppendorf, Germany) consisted of initial denaturation at 94°C/3 min, 35 cycles of 94°C/1 min, T_A (as per Supplementary material, Table S1) x 30 sec, 72°C/1 min and a final extension of 72°C/10 min. Amplification success was visualised on agarose gels containing SYBR Safe DNA gel stain (ThermoFisher Scientific, USA).

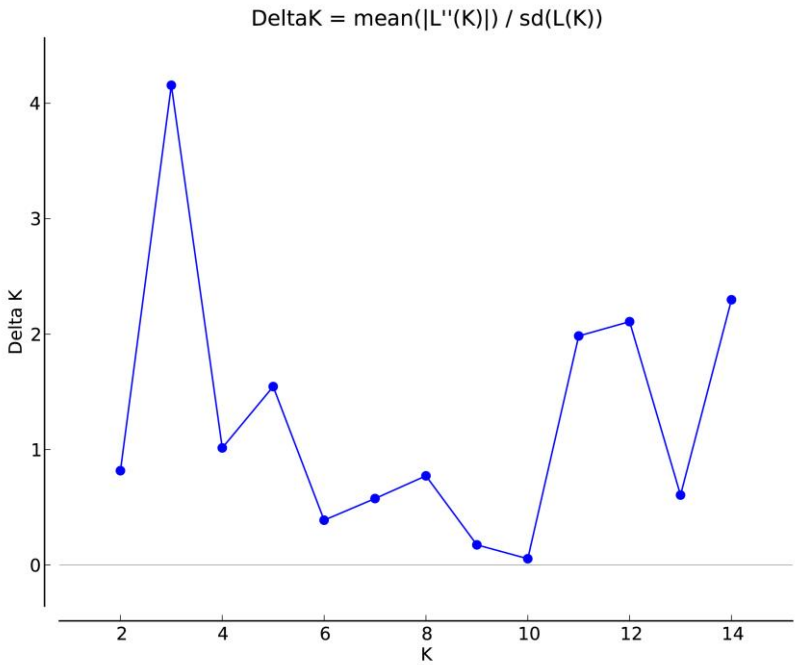
Twelve loci successfully amplified and forward primers for these loci were labelled with proprietary fluorophore dyes; 6-FAM, VIC, NED, PET (Applied Biosystems, USA). Loci were pooled into four PCR multiplex sets based on fragment size and fluorophore (Supplementary material, Table

S1). Following PCR amplifications in each of the *C. albimarginatus* individuals (including labelled primers and as per PCR conditions above), GeneScan™ 500 LIZ ™ size standard (Thermofisher Scientific) and formamide were added to 3 μL of each PCR reaction and 20 μL sample volumes were run on an ABI 3130XL AutoDNA sequencer (Thermofisher). Genotypes were scored and checked by eye using Geneious© R8.1.4 Microsatellite plug-in program (Biomatters Ltd).

The final 12 loci were characterised and found to be polymorphic among 117 individuals of *C. albimarginatus* from four locations within the Indo-Pacific Ocean. Primer details for these loci have been submitted to GenBank (Accessions KY996371 - KY996382). Microsatellite loci were then used for population genetic analysis on 123 individuals from three locations; Seychelles, PNG and Australia. Summary statistics from population genetic analysis including the number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, inbreeding coefficient (F_{IS}), deviations from HWE (HWE*p*) and presence of null alleles are presented in Table S3.

B) Appendix II. Supplementary material from chapter 3; Genetic connectivity of the scalloped hammerhead (*Sphyrna lewini*) in the Pacific and Indian Oceans using a multi-marker approach

a) Tables and Figures



K			Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	8	-12682.85	0.358569	—	—		—	
2	8	-12789.8125	19.086602	-106.9625	15.6		0.817327	
3	8	-12912.375	50.784299	-122.5625	211.0375		4.155566	
4	8	-13245.975	107.589362	-333.6	109.15		1.014506	
5	8	-13470.425	247.389368	-224.45	382.4375		1.545893	
6	8	-14077.3125	663.527188	-606.8875	257.825		0.388567	
7	8	-14942.025	993.000669	-864.7125	571.9375		0.575969	
8	8	-15234.8	854.684298	-292.775	660.35		0.772624	
9	8	-16187.925	650.819968	-953.125	113.85		0.174933	
10	8	-17254.9	1134.342601	-1066.975	62.0375		0.05469	
11	8	-18259.8375	1642.310437	-1004.9375	3258.4625		1.984072	
12	8	-16006.3125	832.74663	2253.525	1755.4875		2.108069	
13	8	-15508.275	660.293051	498.0375	400.2625		0.606189	
14	8	-15410.5	605.650649	97.775	1391.625		2.297736	
15	2	-16704.35	1441.012909	-1293.85	—		—	

Figure B.1. Outputs from microsatellite STRUCTURE analysis showing Evanno's Delta K value (above), a method based on the rate of change in log probability of data and Evanno table output for K = 1-15 (below).

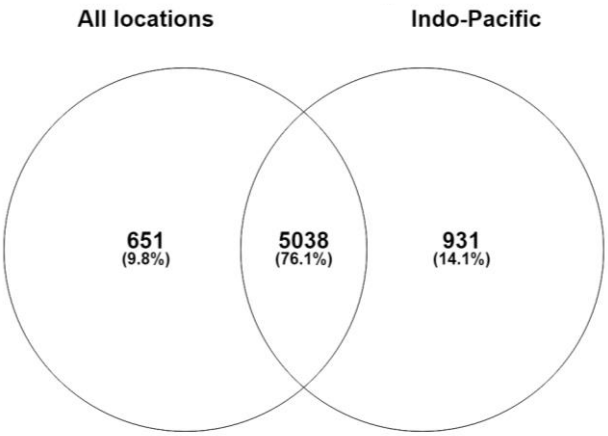


Figure B.2. Comparison between loci filtered for two SNP subsets created; all locations

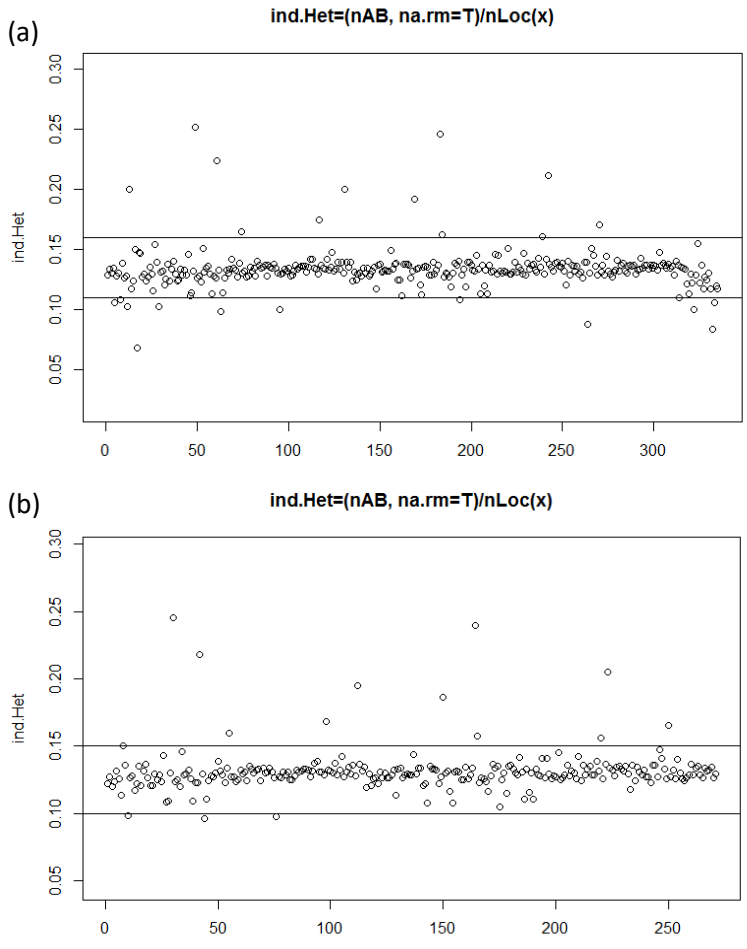


Figure B.3. Average heterozygosity of SNP loci per individual during filtering process of all locations (a) and central Indo-Pacific locations (b). Dashed lines represent cut off range in the SNP filtering process. Heterozygosity was filtered to remove potential individuals of poor DNA quality or sample contamination. Thresholds were selected to remove individuals outside average range for the SNP dataset.

Table B.1. Allele frequncies for 9 microsatellite loci (Nance et al., 2009) genotyped in 12 populations for *S. lewini*.

SLE018	SEY	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ	HAW	GOC
204	-	-	-	0.019	-	-	-	-	-	-	-	-
212	0.038	0.121	0.065	0.093	0.076	0.041	0.138	0.151	0.033	0.091	0.036	0.056
214	0.288	0.121	0.130	0.185	0.121	0.176	0.224	0.093	0.250	0.114	0.107	0.259
216	-	-	-	-	0.030	0.014	-	0.035	0.017	0.023	-	-
224	0.019	-	-	-	-	-	0.017	-	0.017	-	-	-
226	0.404	0.517	0.609	0.556	0.515	0.459	0.345	0.442	0.533	0.545	0.571	0.519
228	0.115	0.172	0.174	0.111	0.167	0.189	0.224	0.198	0.133	0.159	0.250	0.093
230	0.038	0.017	0.022	-	0.030	0.027	-	0.058	0.017	0.023	-	-
232	-	-	-	-	-	0.014	-	-	-	-	-	-
234	-	-	-	0.037	-	0.014	-	-	-	-	-	-
262	0.019	0.017	-	-	0.030	0.014	0.052	0.023	-	0.045	0.036	0.074
SLE027	SEY	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ	HAW	GOC
408	-	-	-	-	-	-	-	-	-	-	0.018	-
416	-	-	-	0.019	-	-	-	-	-	-	-	-
420	0.231	0.224	0.239	0.167	0.227	0.108	0.190	0.198	0.167	0.136	0.036	0.222
422	-	0.017	-	-	-	0.027	-	-	0.017	0.023	-	0.019
424	0.019	-	-	-	-	-	-	-	-	-	-	-
428	0.019	-	-	-	-	-	-	-	-	-	-	-
430	0.096	0.086	0.174	0.111	0.136	0.162	0.121	0.116	0.150	0.182	0.214	-
432	-	0.017	-	-	0.015	-	-	-	-	-	-	-
434	-	-	-	-	-	-	-	-	-	-	-	-
438	-	0.017	0.022	-	0.045	-	0.017	0.012	-	0.045	0.054	-
440	0.192	0.362	0.283	0.315	0.424	0.338	0.310	0.430	0.283	0.455	0.464	0.574
442	0.077	0.103	0.065	-	-	0.068	0.086	0.070	0.100	0.023	0.054	0.019
444	-	-	-	0.037	-	-	-	-	-	-	0.018	-
450	-	-	-	-	0.015	-	-	-	-	-	-	-
452	-	-	-	-	-	-	-	0.012	-	-	0.018	-
454	0.019	-	0.065	-	-	0.014	-	-	-	-	-	-
456	0.212	0.103	0.130	0.204	0.106	0.162	0.172	0.151	0.233	0.091	0.125	0.130
458	0.058	-	0.022	-	-	0.041	0.034	0.012	-	0.045	-	-
476	-	-	-	-	-	-	-	-	0.017	-	-	-

SLE038	SEY	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ	HAW	GOC
392	-	-	-	0.019	-	0.014	0.017	0.012	-	-	-	-
410	-	-	-	-	0.015	-	-	0.023	-	-	-	-
412	-	-	-	-	-	-	-	-	0.017	-	-	-
414	-	-	-	-	-	-	-	-	0.017	-	-	-
416	-	0.017	0.022	-	0.030	0.014	0.017	0.012	0.017	0.023	-	-
418	0.192	0.138	0.130	0.130	0.258	0.216	0.259	0.233	0.300	0.159	0.143	0.278
420	-	-	-	-	0.015	-	0.017	0.012	-	-	0.018	-
422	0.058	0.034	-	0.019	0.045	0.027	0.086	0.012	-	0.045	0.089	0.037
424	0.096	0.121	0.065	0.130	0.045	0.149	0.138	0.151	0.117	0.227	0.054	0.037
426	-	-	-	0.037	-	-	-	-	-	-	-	-
438	-	-	-	-	-	-	-	-	0.017	-	-	-
440	-	0.017	-	-	-	-	-	-	-	-	-	-
442	-	-	-	-	-	-	-	-	0.017	-	-	-
444	-	-	-	-	-	-	-	-	-	0.045	-	-
446	0.173	0.103	0.043	0.056	0.167	0.135	0.086	0.116	0.200	0.159	0.107	0.074
448	-	-	-	-	-	-	-	0.012	-	-	0.018	0.056
450	0.077	0.086	0.043	0.093	0.121	0.108	0.121	0.128	0.100	0.091	0.071	0.019
452	0.154	0.190	0.043	0.296	0.227	0.257	0.207	0.256	0.183	0.159	0.429	0.315
454	-	-	-	0.019	0.015	0.014	0.017	-	0.017	0.045	-	-
456	-	-	-	-	-	0.014	-	-	-	-	-	-
470	0.019	-	-	-	-	-	-	-	-	-	-	-
472	0.019	-	-	-	-	-	-	-	-	-	-	-
476	0.019	-	-	0.019	-	0.027	-	-	-	-	-	-
478	-	0.017	-	-	0.030	-	-	0.035	-	0.045	-	-
480	-	-	-	0.037	-	-	-	-	-	-	-	-

SLE045	SEY	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ	HAW	GOC
398	-	-	-	-	-	0.014	-	-	-	-	-	-
402	-	0.017	0.022	-	0.015	0.041	0.034	0.023	0.017	0.023	-	0.148
404	0.154	0.224	0.087	0.037	0.106	0.068	0.138	0.081	0.133	0.136	0.286	0.111
406	0.058	0.034	-	0.056	0.030	0.081	0.017	0.070	0.083	0.091	-	-
408	0.404	0.328	0.522	0.352	0.394	0.351	0.448	0.407	0.483	0.341	0.357	0.333
410	0.308	0.328	0.326	0.481	0.455	0.392	0.328	0.419	0.283	0.409	0.357	0.389
412	-	-	-	-	-	-	-	-	-	-	-	0.019

SLE053	SEY	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ	HAW	GOC
387	0.038	0.017	-	-	-	0.014	-	-	-	0.023	-	-
403	-	-	-	0.019	-	-	-	-	-	-	-	-
411	-	0.034	-	-	0.030	-	0.017	0.035	-	-	-	0.074
421	0.038	0.034	0.022	0.037	-	0.041	-	-	0.033	0.045	-	-
423	0.019	-	-	-	0.015	0.014	0.017	0.012	-	-	-	-
425	0.019	-	-	-	-	-	-	0.012	-	-	-	-
427	0.019	-	0.022	-	-	0.027	0.052	0.023	0.017	-	0.036	-
431	0.038	-	0.022	0.019	-	-	0.017	-	0.017	0.045	0.018	-
433	0.077	0.138	0.065	0.130	0.106	0.176	0.034	0.116	0.133	0.023	0.161	0.500
435	-	-	0.022	0.019	0.030	0.014	0.017	0.035	0.017	0.023	-	-
437	-	-	0.022	-	0.030	0.014	0.103	-	-	-	-	-
439	0.019	-	-	0.037	0.030	0.041	0.017	0.047	0.050	0.023	-	-
441	0.096	0.069	0.022	0.056	0.045	0.081	0.052	0.116	0.050	0.023	-	0.037
443	0.096	0.155	0.109	0.130	0.182	0.122	0.121	0.163	0.233	0.227	0.161	0.111
445	0.058	0.034	0.022	0.093	0.045	0.027	0.086	0.035	0.050	0.068	-	-
447	0.058	0.034	0.065	0.019	0.045	0.081	0.034	0.012	0.017	0.045	0.018	-
449	0.212	0.155	0.130	0.074	0.152	0.108	0.224	0.140	0.167	0.136	0.286	0.093
451	0.096	0.052	0.109	0.056	0.091	0.095	0.069	0.093	0.100	0.045	0.054	0.037
453	0.038	0.121	0.065	0.148	0.182	0.068	0.138	0.128	0.117	0.159	0.089	0.111
455	-	0.034	-	0.019	-	-	-	-	-	0.045	-	-
457	-	0.017	-	-	0.015	-	-	-	-	0.023	-	-
459	-	-	-	-	-	-	-	0.012	-	-	-	-

SLE081	SEY	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ	HAW	GOC
376	-	0.017	-	0.019	0.015	-	-	0.012	-	-	-	-
378	-	-	-	-	-	0.014	-	-	-	-	-	-
386	-	-	-	-	-	0.014	-	-	-	-	-	-
396	0.019	-	-	-	-	0.027	-	-	-	0.023	0.036	0.019
398	-	0.017	-	-	0.045	-	0.034	0.023	0.017	0.023	0.054	0.019
400	-	0.017	0.022	-	0.015	0.041	-	-	-	-	-	-
402	0.269	0.121	0.043	0.278	0.242	0.257	0.172	0.198	0.233	0.205	0.250	0.167
404	0.135	0.034	0.043	0.056	0.076	0.068	0.138	0.209	0.117	0.159	0.179	0.148
406	0.115	0.103	0.022	0.185	0.152	0.270	0.224	0.105	0.183	0.136	0.107	0.204
408	0.173	0.276	0.043	0.222	0.197	0.162	0.207	0.244	0.217	0.227	0.125	0.148

410	0.058	0.121	0.043	0.074	0.091	0.054	0.138	0.058	0.083	0.091	0.018	0.093
412	-	0.052	0.022	0.074	0.045	0.027	0.017	-	0.083	0.023	0.018	-
414	0.077	0.052	0.022	0.093	0.076	0.068	0.052	0.116	0.067	0.091	-	0.019
416	-	0.034	-	-	0.015	-	-	0.012	-	0.023	-	-
418	-	-	-	-	-	-	0.017	-	-	-	-	-
420	-	0.017	-	-	-	-	-	-	-	-	-	-

SLE071	SEY	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ	HAW	GOC
231	-	-	-	-	0.030	-	-	0.035	0.017	-	0.018	0.019
233	0.019	-	0.022	0.019	0.030	-	0.052	0.035	0.033	0.045	0.018	0.019
251	0.385	0.190	-	0.056	0.242	0.216	0.069	0.151	0.083	0.318	0.250	0.074
253	-	-	-	-	0.015	-	0.034	-	-	0.045	-	-
255	0.096	0.052	0.043	0.056	0.030	0.054	0.052	0.070	0.050	0.045	0.018	0.019
257	0.058	0.017	-	-	-	0.014	-	-	-	-	0.036	-
259	-	-	-	-	-	-	-	-	-	-	0.018	-
267	-	-	-	-	-	-	-	-	0.017	-	-	-
269	-	-	-	0.037	0.015	0.027	-	-	0.017	-	0.036	-
271	0.269	0.517	0.717	0.593	0.470	0.486	0.655	0.512	0.500	0.364	0.339	0.611
273	-	-	-	-	0.030	-	-	0.023	-	-	0.018	0.019
277	0.019	0.034	-	-	0.015	0.041	-	0.058	0.083	0.023	0.054	0.130
279	0.038	0.017	-	0.019	0.076	0.081	0.069	0.023	0.050	-	0.107	0.019
281	-	-	-	-	0.015	0.014	-	0.012	-	0.023	-	-
283	-	-	-	0.019	0.030	-	-	-	-	-	0.018	0.019
285	-	-	-	0.019	-	-	-	0.012	0.033	-	-	0.037
287	-	-	-	0.037	-	-	-	-	-	-	-	-
289	-	-	-	0.037	-	0.027	-	-	-	-	-	-
291	-	-	-	0.037	-	0.014	-	-	-	-	-	-
293	-	0.069	-	-	-	-	-	-	0.017	-	-	-

SLE077	SEY	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ	HAW	GOC
188	-	-	-	-	-	0.014	-	-	-	-	-	-
206	-	-	-	-	-	0.014	-	-	-	-	-	-
216	-	-	-	-	-	-	-	-	-	0.023	-	-
220	-	-	-	-	0.015	-	-	-	-	-	-	-
222	0.019	0.034	-	-	0.015	-	0.017	-	-	-	-	-
224	-	-	0.022	-	-	-	-	-	0.017	-	-	-

226	0.038	-	-	-	-	-	0.034	-	-	-	-	-
228	-	-	-	0.019	-	-	0.017	-	0.017	-	0.018	-
230	0.038	0.017	-	0.019	-	0.014	0.017	-	-	0.045	-	-
232	0.019	0.052	0.065	0.056	0.045	0.068	0.034	0.070	0.083	0.023	0.143	0.093
234	0.019	-	0.087	-	-	0.041	-	0.035	0.017	0.068	0.018	-
236	0.038	0.017	0.087	0.037	0.015	-	0.052	0.012	0.017	-	0.018	-
238	0.058	0.069	0.022	0.074	0.045	0.041	0.017	0.058	0.067	0.045	0.018	0.130
240	0.019	0.086	0.043	0.074	0.015	0.041	0.017	0.023	0.083	0.068	0.054	-
242	0.019	0.052	0.109	0.074	0.045	0.054	0.069	0.035	0.033	0.023	0.071	-
244	0.058	0.052	0.022	0.093	0.076	0.135	0.103	0.151	0.100	0.136	0.214	0.241
246	0.058	0.172	0.087	0.093	0.152	0.054	0.086	0.128	0.100	0.091	0.054	-
248	0.038	0.069	0.022	0.037	0.045	0.068	0.069	0.035	0.033	0.136	0.036	0.167
250	0.038	0.103	0.065	0.074	0.061	0.108	0.069	0.035	0.050	0.045	0.054	0.056
252	0.038	0.069	0.130	-	0.030	0.014	0.069	0.070	0.050	0.068	0.071	-
254	0.077	-	0.022	0.019	0.015	0.027	0.017	0.070	-	-	-	0.019
256	0.038	-	-	-	0.045	0.041	0.034	0.047	0.017	-	-	0.019
258	0.077	-	0.043	-	0.015	0.054	-	0.035	0.033	-	0.054	-
260	0.038	0.034	0.043	-	0.030	-	0.017	-	0.050	0.045	-	-
262	0.019	0.017	0.022	0.037	-	-	0.017	0.012	0.033	-	-	0.019
264	0.058	-	-	0.019	-	-	0.017	0.035	-	-	-	-
266	0.038	-	-	0.019	0.015	-	0.034	-	-	-	0.018	-
268	-	0.017	-	-	-	0.014	0.017	-	-	0.023	-	0.019
270	-	0.017	-	-	0.030	0.014	0.034	0.012	-	0.045	0.018	-
272	-	-	0.022	-	-	0.014	-	0.012	-	-	-	-
274	0.019	-	-	-	-	-	0.034	0.023	0.033	-	-	-
276	-	-	-	0.019	-	-	0.017	-	0.033	-	-	0.019
278	-	-	-	-	-	0.014	-	0.012	-	0.023	-	-
282	-	-	0.022	0.019	-	-	-	-	-	-	-	0.019
284	-	0.034	-	0.037	-	0.027	-	0.012	-	-	-	-
286	-	-	-	0.019	0.015	-	-	-	-	-	-	-
288	-	-	-	-	-	-	-	-	0.017	-	-	-
290	-	0.017	-	-	-	-	0.034	0.012	0.017	-	-	-
294	-	-	-	-	0.015	-	-	-	-	0.023	-	-
296	-	0.017	-	-	0.015	0.014	0.017	-	-	-	0.018	-
300	0.058	-	0.022	-	-	-	0.017	-	-	-	-	-

312	-	0.017	-	-	-	-	-	-	-	-	-	-
316	-	-	-	0.019	-	0.014	-	-	0.017	-	0.036	-
318	-	-	-	0.019	-	-	-	-	-	-	-	-
322	-	-	-	-	-	-	0.017	0.012	-	-	-	-
324	-	-	-	0.019	-	-	-	-	-	0.045	-	-
326	-	-	-	0.019	-	0.027	-	-	-	-	-	-
328	-	0.017	-	0.056	-	0.041	-	0.012	0.083	0.023	0.036	0.130
330	-	0.017	-	-	-	0.014	-	-	-	-	-	-
334	-	-	-	-	-	-	-	-	-	-	0.018	-
336	-	-	-	-	-	-	-	-	-	-	-	0.019
350	-	-	-	-	-	-	-	-	-	-	-	0.019

SLE089	SEY	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ	HAW	GOC
172	-	0.017	0.022	-	-	-	-	-	-	0.023	-	0.019
174	-	0.034	0.022	-	-	-	-	0.012	0.033	-	0.018	-
178	-	-	-	-	-	0.014	-	-	-	-	-	0.019
180	0.019	-	-	0.111	-	0.054	0.017	-	0.050	-	-	-
182	0.115	0.017	0.130	-	0.030	0.027	0.052	0.058	-	0.159	0.054	0.056
184	-	-	-	-	-	0.014	-	-	-	-	-	-
186	0.115	0.086	0.130	0.019	0.091	0.081	0.103	0.023	0.067	0.045	0.071	0.074
188	0.058	0.138	0.043	0.056	0.030	0.041	0.069	0.070	0.100	0.114	0.054	0.074
190	0.192	0.103	0.130	0.130	0.152	0.149	0.121	0.128	0.100	0.068	0.161	0.278
192	0.115	0.086	0.022	0.130	0.106	0.041	0.103	0.070	0.083	0.114	0.179	0.074
194	0.096	0.155	0.152	0.222	0.152	0.122	0.190	0.279	0.133	0.114	0.214	0.278
196	0.115	0.172	0.043	0.056	0.212	0.122	0.138	0.221	0.117	0.205	0.107	0.037
198	-	0.103	0.152	0.056	0.121	0.108	0.017	0.093	0.117	0.068	0.036	-
200	0.019	0.017	-	0.056	0.015	0.027	0.017	0.023	0.033	0.023	0.089	0.074
202	0.038	0.017	0.043	-	0.015	0.027	-	-	0.033	0.045	-	-
204	-	-	-	-	0.015	-	-	-	-	-	-	-
210	-	0.017	-	0.019	0.015	0.095	0.069	0.023	0.033	0.023	0.018	0.019
212	-	-	-	-	0.015	-	-	-	-	-	-	-

Table B.2. Filtering processes of SNPs for two population scenarios. All locations and central Indo-Pacific locations (PHTW, IN, WA, NT, PNG, PCB, TSV, NSW, FJ).

All locations		
	SNPs remaining	Individuals remaining
Initial SNPS	53,729	
Initial Individuals		352
DaRT Reproducibility (> 0.99)	38,304	
Remove monomorphic SNPs	37,129	
Retain call rate per ind. (> 0.70)		335
Remove monomorphic SNPs	27,411	
Retain call rate per SNP (> 0.95)	17,384	
Remove SNPs with Minor Allele Frequency (< 0.02)	7,452	
Retain SNPs with Heterozygosity per individual (between 0.11-0.16)		310
Remove SNPS with HWE (< 0.05)	5,706	
Remove monomorphic SNPs	5,689	
TOTAL	5,689	310

Central Indo-Pacific		
	SNPs remaining	Individuals remaining
Initial SNPS	53,729	
Initial Individuals		281
DaRT Reproducibility (> 0.99)	38,304	
Remove monomorphic SNPs	34,631	
Retain call rate per ind. (> 0.80)		271
Remove monomorphic SNPs	25,878	
Retain call rate per SNP (> 0.95)	17,183	
Remove SNPs with Minor Allele Frequency (< 0.02)	7,827	
Retain SNPs with Heterozygosity per individual (between 0.10-0.15)		265
Remove SNPS with HWE (< 0.05)	5,969	
TOTAL	5,969	265

Table B.3. Polymorphic nucleotide positions in mtDNA control region of *S. lewini* showing the similarity between Atlantic haplotype as described in Quattro et al., (2006) and individuals from SEY- Seychelles, IN- Indonesia, PH- Philippines and PNG- Papua New Guinea. Nucleotides shared with the Atlantic haplotype are indicated with a period or otherwise stated. Insertion is represented by a dash '-'.

	Nucleotide position														
Haplotypes	133	141	172	215	218	225	237	257	259	262	265	280	281	310	393
Atlantic*	A	A	T	T	T	C	G	C	A	-	C	C	T	C	A
SEY (n = 18)	.	.	A	.	.	.	.	.	.	-	.	.	.	.	.
SEY (n = 3)	.	.	A	.	.	.	.	.	.	-	.	.	.	T	.
SEY (n = 1)	.	.	A	G	.	.	.	.	.	-	.	.	.	.	.
IN (n = 1)	.	.	A	.	.	.	.	.	.	-	.	.	C	.	.
PH (n = 3)	.	.	A	.	.	.	.	.	.	-	.	.	C	.	.
PNG (n = 1)	.	.	A	.	.	.	.	.	.	-	.	.	C	.	.
Indo-Pacific*	C	T	A	T	A	T	A	T	G	T	T	T	C	T	G

*nucleotide position and haplotypes described in Quattro et al., (2006)

Table B.4. Summary statistics for microsatellite loci per *S. lewini* population.

Locations	Parameter	SLE027	SLE089	SLE018	SLE081	SLE077	SLE071	SLE053	SLE038	SLE045
SEY (n = 26)										
	n	24	23	24	22	24	23	24	21	24
	H_O	0.833	0.870	0.542	0.773	0.917	0.522	0.750	0.952	0.750
	H_E	0.819	0.866	0.691	0.800	0.948	0.699	0.891	0.831	0.666
	F_{IS}	-0.020	0.000	0.220	0.030	0.03	0.25	0.16	-0.15	-0.13
	HWE*p*	0.462	0.683	0.448	0.742	0.385	0.046	0.122	0.752	1.000
	Null Allele						*	*		
PHTW (n = 29)										
	n	27	28	28	25	29	26	26	21	27
	H_O	0.852	0.929	0.464	0.840	0.897	0.269	0.538	0.857	0.630
	H_E	0.757	0.880	0.649	0.832	0.923	0.611	0.881	0.829	0.693
	F_{IS}	-0.13	-0.06	0.28	-0.01	0.03	0.56	0.39	-0.03	0.09
	HWE*p*	0.890	0.669	0.025	0.617	0.071	**0.000**	**0.000**	0.752	0.721
	Null Allele			*			*	*		
IN (n = 23)										
	n	23	21	23	6	22	18	16	8	22
	H_O	0.739	0.762	0.304	0.833	0.773	0.111	0.750	0.750	0.500
	H_E	0.806	0.874	0.578	0.861	0.924	0.156	0.883	0.773	0.577
	F_{IS}	0.08	0.13	0.47	0.03	0.16	0.29	0.15	0.03	0.13
	HWE*p*	0.539	0.183	**0.002**	0.397	**0.005**	0.086	0.123	0.469	0.427
	Null Allele			*		*				

WA (n = 27)										
	n	23	23	27	27	26	25	23	23	25
	H_O	0.739	0.652	0.333	0.815	0.846	0.240	0.783	0.696	0.400
	H_E	0.749	0.851	0.634	0.816	0.941	0.575	0.889	0.811	0.580
	F_{IS}	0.01	0.23	0.47	0.00	0.10	0.58	0.12	0.14	0.31
	HWEp	0.437	**0.002**	**0.000**	0.399	0.039	**0.000**	0.469	0.152	0.055
	Null Allele		*	*			*			
NT (n = 33)										
	n	32	32	32	32	25	33	33	32	33
	H_O	0.719	0.750	0.469	0.813	0.880	0.697	0.788	0.813	0.758
	H_E	0.719	0.864	0.664	0.846	0.917	0.709	0.881	0.822	0.626
	F_{IS}	0.00	0.13	0.29	0.04	0.04	0.02	0.11	0.01	-0.21
	HWE*p*	0.235	0.498	0.013	0.053	0.464	0.159	0.111	0.483	0.118
	Null Allele		*	*			*			
PNG (n = 37)										
	n	34	34	35	37	36	36	34	36	35
	H_O	0.676	0.912	0.486	0.973	0.917	0.694	0.765	0.750	0.543
	H_E	0.780	0.896	0.686	0.819	0.936	0.687	0.894	0.824	0.676
	F_{IS}	0.13	-0.02	0.29	-0.19	0.02	-0.01	0.14	0.09	0.20
	HWE*p*	0.334	0.089	0.022	0.523	0.702	0.023	**0.003**	0.036	0.102
	Null Allele			*				*		

PCB (n = 29)										
	n	27	26	29	29	29	27	29	28	28
	H_O	0.815	0.808	0.448	0.793	0.966	0.333	0.759	0.821	0.714
	H_E	0.786	0.871	0.759	0.835	0.948	0.486	0.884	0.829	0.647
	F_{IS}	-0.04	0.07	0.41	0.05	-0.02	0.31	0.14	0.01	-0.10
	HWE*p*	0.605	0.570	**0.001**	0.384	0.757	0.033	0.099	0.589	0.613
	Null Allele			*			*			
TSV (n = 43)										
	n	43	43	43	42	41	40	42	43	43
	H_O	0.698	0.814	0.535	0.833	0.951	0.375	0.643	0.767	0.558
	H_E	0.734	0.833	0.729	0.821	0.924	0.657	0.890	0.825	0.647
	F_{IS}	0.05	0.02	0.27	-0.02	-0.03	0.43	0.28	0.07	0.14
	HWE*p*	0.044	0.267	**0.000**	0.678	0.322	**0.000**	0.006	0.376	0.479
	Null Allele			*			*	*		
NSW (n = 30)										
	n	29	27	30	30	30	27	30	30	30
	H_O	0.793	0.963	0.433	0.833	0.900	0.519	0.767	0.867	0.567
	H_E	0.791	0.897	0.633	0.833	0.938	0.664	0.867	0.811	0.661
	F_{IS}	0.00	-0.07	0.32	0.00	0.04	0.22	0.12	-0.07	0.14
	HWE*p*	0.846	0.757	0.023	0.363	0.163	0.072	0.412	0.145	0.017
	Null Allele			*						

FJ (n = 22)										
	n	22	22	22	22	22	19	21	22	22
	H_O	0.773	0.773	0.636	0.909	0.864	0.579	0.810	0.909	0.682
	H_E	0.728	0.879	0.653	0.844	0.925	0.677	0.875	0.855	0.689
	F_{IS}	-0.06	0.12	0.03	-0.08	0.07	0.15	0.08	-0.06	0.01
	HWE*p*	0.448	0.060	0.524	0.922	0.419	0.107	0.081	0.234	0.656
	Null Allele									
HAW (n = 28)										
	n	28	28	28	22	27	26	23	26	28
	H_O	0.714	0.857	0.750	0.864	0.889	0.731	0.826	0.808	0.643
	H_E	0.715	0.864	0.597	0.795	0.898	0.772	0.784	0.731	0.663
	F_{IS}	0.00	0.01	-0.26	-0.09	0.01	0.05	-0.05	-0.11	0.03
	HWE*p*	0.428	0.393	0.734	0.764	0.109	0.069	0.254	0.805	0.963
	Null Allele									
GoC (n = 27)										
	n	26	27	27	22	26	26	26	22	27
	H_O	0.692	0.852	0.519	0.727	0.654	0.385	0.846	0.682	0.704
	H_E	0.572	0.818	0.647	0.815	0.856	0.570	0.686	0.717	0.703
	F_{IS}	-0.21	-0.04	0.20	0.11	0.24	0.32	-0.23	0.05	0.00
	HWE*p*	0.763	0.038	0.054	0.366	0.026	**0.001**	0.720	0.549	0.815
	Null Allele					*	*			

The table describes the following parameters per microsatellite locus for each location; observed (H_O) and expected (H_E) heterozygosity, inbreeding coefficient (F_{IS}), Hardy-Weinberg Equilibrium p value (HWE*p*) following Bonferroni correction ($p < 0.001$). Asterisks indicate potential presence of null alleles as indicated by MICROCHECKER.

Table B.5. Results of power analysis conducted in POWSIM for microsatellites and SNPs (all location set, 5689 SNPs). Time in generations (t), effective population size of subpopulations (N*e*).

9 Microsatellites				5,689 SNPs			
F_{ST}	t	N*e*	Power	F_{ST}	t	N*e*	Power
0.05	100	1000	1.00	0.05	100	1000	1.00
0.02	50	1000	1.00	0.02	50	1000	1.00
0.01	20	1000	1.00	0.01	20	1000	1.00
0.004	10	1000	1.00	0.004	10	1000	1.00
0.001	2	1000	1.00	0.001	2	1000	1.00

Table B.6. Microsatellite and SNP pairwise genetic differences (F_{ST}) as displayed in figure 3. Calculated using 9 microsatellite loci and 5,689 SNP loci across all populations.

	Microsatellites											
	SEY	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ	HAW	GOC
SEY	*	0.016	**0.001**	**0.000**	0.033	0.032	0.003	0.002	0.029	0.059	**0.001**	**0.000**
PHTW	0.016	*	0.306	0.353	1.000	0.490	0.400	0.820	0.787	0.696	0.064	**0.000**
IN	0.036	0.005	*	0.395	0.768	0.205	0.918	0.300	0.901	0.011	**0.000**	**0.000**
WA	0.032	0.004	0.004	*	0.921	0.504	0.297	0.755	0.799	0.109	0.002	0.002
NT	0.011	0.000	0.000	0.000	*	0.968	0.504	0.998	0.728	0.999	0.341	**0.000**
PNG	0.011	0.001	0.006	0.001	0.000	*	0.132	0.588	0.722	0.452	0.046	**0.000**
PCB	0.020	0.002	0.000	0.005	0.000	0.006	*	0.407	0.329	0.018	0.002	**0.000**
TSV	0.020	0.000	0.005	0.000	0.000	0.000	0.002	*	0.248	0.356	0.011	**0.000**
NSW	0.013	0.000	0.000	0.000	0.000	0.000	0.003	0.004	*	0.089	0.027	**0.000**
FJ	0.010	0.000	0.021	0.010	0.000	0.001	0.015	0.003	0.009	*	0.250	**0.000**
HAW	0.021	0.009	0.035	0.022	0.001	0.008	0.019	0.012	0.011	0.003	*	**0.000**
GOC	0.064	0.033	0.044	0.025	0.032	0.029	0.041	0.030	0.034	0.047	0.039	*
						SNPs						
SEY	*	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
PHTW	0.014	*	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
IN	0.012	0.004	*	**0.000**	0.016	**0.000**	0.005	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**
WA	0.012	0.005	0.004	*	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
NT	0.012	0.004	0.001	0.002	*	0.004	0.103	0.002	**0.001**	**0.000**	**0.000**	**0.000**
PNG	0.012	0.004	0.002	0.003	0.001	*	0.043	0.003	0.035	**0.000**	**0.000**	**0.000**
PCB	0.009	0.005	0.002	0.003	0.001	0.001	*	0.023	0.084	**0.000**	**0.000**	**0.000**
TSV	0.011	0.004	0.002	0.003	0.001	0.001	0.001	*	**0.000**	**0.000**	**0.000**	**0.000**
NSW	0.012	0.004	0.002	0.003	0.002	0.001	0.001	0.002	*	**0.000**	**0.000**	**0.000**
FJ	0.013	0.009	0.007	0.008	0.007	0.004	0.005	0.005	0.005	*	**0.000**	**0.000**
HAW	0.023	0.016	0.017	0.016	0.017	0.014	0.015	0.014	0.014	0.012	*	**0.000**
GOC	0.072	0.048	0.05	0.045	0.046	0.046	0.049	0.047	0.044	0.052	0.047	*

Above diagonal; *p* values, below diagonal; pairwise F_{ST} values, significant values (p = < 0.001) are in bold.

Table B.7. Central Indo-Pacific pairwise genetic differences (F_{ST}) calculated using SNP loci (5969 SNPs).

	PHTW	IN	WA	NT	PNG	PCB	TSV	NSW	FJ
PHTW	*	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
IN	0.005	*	**0.000**	0.020	**0.000**	0.012	**0.000**	**0.000**	**0.000**
WA	0.005	0.004	*		**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
NT	0.004	0.001	0.002	*	**0.000**	0.041	**0.001**	**0.000**	**0.000**
PNG	0.003	0.002	0.003	0.001	*	0.088	**0.000**	0.004	**0.000**
PCB	0.004	0.002	0.003	0.001	0.001	*	0.136	0.130	**0.000**
TSV	0.004	0.003	0.003	0.001	0.001	0.001	*	**0.000**	**0.000**
NSW	0.004	0.002	0.003	0.002	0.001	0.001	0.002	*	**0.000**
FJ	0.010	0.008	0.009	0.007	0.005	0.004	0.006	0.005	*

Above diagonal; *p* values, below diagonal; pairwise F_{ST} values, significant values (p = < 0.001) are in bold.

b) Supplementary Methods

SNP filtering and processing

A total of 352 individuals from 12 regions were sent to DArT Pty Ltd. for GBS DArTSeq processing. DArTSeq returned a total dataset of 53,729 SNPs. To test for global and fine scale structure using SNPs we ran filtering and quality control on two different datasets. The first included all twelve locations; SEY, PHTW, IN, WA, NT, PNG, PCB, TSV, NSW, FJ, HAW and GOC, while the second dataset consisted of closely located regions within the Central Indo-Pacific (CIP); PHTW, IN, WA, NT, PNG, PCB, TSV, NSW and FJ.

The total dataset (all locations) of 352 individuals and all twelve locations was filtered according to the following criteria: (a) only one SNP per tag, (b) DArT Reproducibility > 0.99, (c) missing data per individual < 0.30, (d) missing data per SNP < 0.05, (e) Minor allele frequency > 0.02, (f) heterozygosity per individual between 0.11-0.16 (due to excessive low and high heterozygosity likely representing poor DNA quality or sample contamination respectively), (g) no loci out of Hardy-Weinberg Equilibrium (Table S2). Filtering resulted in a set of 5,689 SNPs across all populations.

Filtering for the CIP dataset (n = 265) was as follows: (a) only one SNP per tag, (b) DArT Reproducibility > 0.99, (c) missing data per individual < 0.20, (d) missing data per SNP < 0.05, (e) Minor allele frequency > 0.02, (f) heterozygosity per individual between 0.02-0.14, (g) no loci out of Hardy-Weinberg Equilibrium (Table S2). Filtering resulted in a final set of 5,969 SNPs for the CIP dataset.

Datasets had relatively similar numbers of SNPs, however SNP loci did slightly differ between groups. To visualise difference in SNPs sets Venny v2.1 was used (Figure S2) (Oliveros 2007). The majority of SNP loci selected for each dataset were the same (4,929), however the CIP did have the most unique SNP loci after filtering (931).

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